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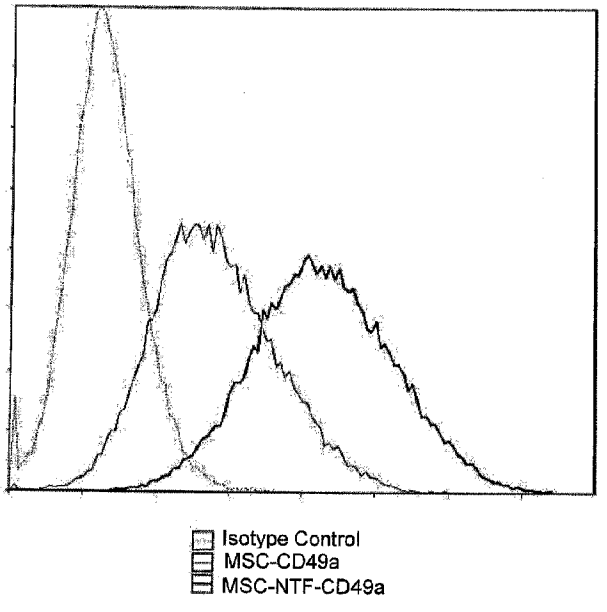
(54) Titre : COMPOSITION PHARMACEUTIQUE COMPRENANT DES CELLULES SOUCHES MESENCHYMATEUSES DERIVEES DE MOELLE OSSEUSE

(54) Title: PHARMACEUTICAL COMPOSITION COMPRISING BONE-MARROW DERIVED MESENCHYMAL STEM CELLS



(57) Abrégé/Abstract:
A method of qualifying whether a cell population is a suitable therapeutic is disclosed. The method comprises: (a) incubating a population of undifferentiated mesenchymal stem cells (MSCs) in a differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP for at least two days to obtain a population of differentiated MSCs; and (b) analyzing the expression of CD49a in the differentiated MSC population, wherein an amount of CD49a above a predetermined level indicative of the cell population being suitable as a therapeutic.

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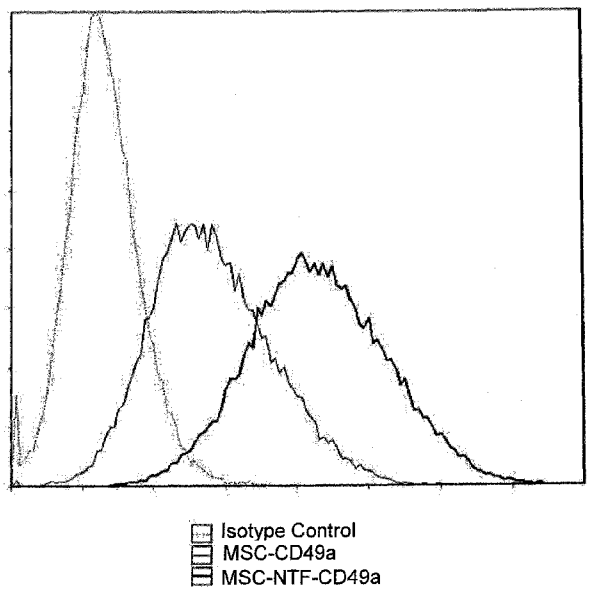
[Continued on next page]

(54) Title: METHOD OF QUALIFYING CELLS

(57) Abstract: A method of qualifying whether a cell population is a suitable therapeutic is disclosed. The method comprises: (a) incubating a population of undifferentiated mesenchymal stem cells (MSCs) in a differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP for at least two days to obtain a population of differentiated MSCs; and (b) analyzing the expression of CD49a in the differentiated MSC population, wherein an amount of CD49a above a predetermined level indicative of the cell population being suitable as a therapeutic.

FIG. 1



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PHARMACEUTICAL COMPOSITION COMPRISING BONE-MARROW DERIVED MESENCHYMAL STEM CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to a method of qualifying neurotrophic factor secreting cells based on cell surface marker expression.

Amyotrophic lateral sclerosis (ALS) is one of the most common neurodegenerative diseases in adults. It is a fatal progressive neurodegenerative disease characterized by motor-neuron cell death in the brain and spinal cord accompanied by rapid loss of muscle function and eventual complete paralysis.

Current experimental ALS drugs are developed on the basis of putative pathophysiologic mechanisms, such as anti-glutamatergic agents, drugs targeting protein misfolding and accumulation, antioxidant therapy, immunomodulatory agents, and stem cells.

Of the current investigational therapies, stem cell transplantation may have the most potential. Apart from the replacement of lost or damaged motor neurons, stem cell implantation therapy may benefit ALS patients by an independent effect of cytoprotection. Further, there is the potential for stem cells to differentiate into supportive interstitial cells including astrocytes and microglia which can potentially produce neurotrophic factors as well as enzymatic and paracrine mediators which antagonize neurotoxicity. Further experimental data have shown that non-neuronal cell replacement can be a strategic therapy in promoting motor neuron survival and improved neuromuscular function (Corti S et al. Brain (2010) 133 (2): 465-481).

The use of stem cells as a cellular source in cell replacement therapy for additional neurodegenerative diseases including Parkinson's disease and multiple sclerosis has also been suggested.

Neurotrophic factors (NTF) are small, naturally occurring polypeptides that support the development and survival of neurons, and therefore have been considered in the past few years as candidates for therapy options for different neurodegenerative diseases including ALS. Studies in ALS animal models have shown a delay in disease onset and/or progression after administration of various neurotrophic factors.

However, clinical trials of systematic or intrathecal administration of recombinant growth factors to ALS patients have not been effective, probably due in

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part to their short half-life, low concentrations at target sites, and high incidence of side effects.

Several studies have shown that mesenchymal stem cells (MSCs) following exposure to different factors *in vitro,* change their phenotype and demonstrate neuronal and glial markers [Kopen, G.C., et al., Proc Natl Acad USA. 96(19):10711-6, 1999; Sanchez-Ramos, et al. Exp Neurol. 164(2):247-56. 2000; Woodbury, D., J Neurosci Res. 61(4):364-70,2000; Woodbury, D., et al., J Neurosci Res. 69(6):908-17, 2002; Black, I.B., Woodbury, D. Blood Cells Mol Dis. 27(3):632-6, 2001; Kohyama, J., et al. Differentiation. 68(4-5):235-44, 2001; Levy, Y.S. J Mol Neurosci. 21(2):121-32, 2003, Blondheim N.R.,. Stem Cells & Dev. 15:141-164, 2006].

WO2006/134602 and WO2009/144718 teaches differentiation protocols for the generation of neurotrophic factor secreting cells from mesenchymal stem cells.

WO2007/066338 teaches differentiation protocols for the generation of oligodendrocyte-like cells from mesenchymal stem cells.

WO2004/046348 teaches differentiation protocols for the generation of neuronal-like cells from mesenchymal stem cells.

WO 2014/024183 teaches additional differentiation protocols for the generation of cells which secrete neurotrophic factors.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of qualifying whether a cell population is a suitable therapeutic comprising:

(a) incubating a population of undifferentiated mesenchymal stem cells (MSCs) in a differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP for at least two days to obtain a population of differentiated MSCs; and

(b) analyzing the expression of CD49a in the differentiated MSC population, wherein an amount of CD49a above a predetermined level indicative of the cell population being suitable as a therapeutic.

According to an aspect of some embodiments of the present invention there is provided an isolated population of mesenchymal stem cells having been ex vivo

differentiated into cells that secrete neurotrophic factors by incubation in a differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP for at least two days to obtain a population of differentiated MSCs, wherein at least 80 % of the cells of the population express CD49a.

According to an aspect of some embodiments of the present invention there is provided a method of treating an immune or inflammatory related disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of mesenchymal stem cells having been ex vivo differentiated into cells that secrete neurotrophic factors, wherein the immune or inflammatory related disease is not a neurodegenerative disease or myasthenia gravis, thereby treating the disease.

According to an aspect of some embodiments of the present invention there is provided a use of mesenchymal stem cells which have been ex vivo differentiated into cells that secrete neurotrophic factors for the treatment of an immune or inflammatory related disease, wherein the immune or inflammatory related disease is not a neurodegenerative disease or myasthenia gravis, thereby treating the disease.

According to some embodiments of the invention, the analyzing the expression of CD49a comprises analyzing the number of cells of the differentiated MSC population which express CD49a, wherein a number of cells being greater than 80 % is indicative of the cell population being suitable as a therapeutic.

According to some embodiments of the invention, analyzing the expression of CD49a comprises analyzing the level of expression of CD49a in said differentiated MSC population, wherein an increase in the level of expression by more than 2 fold compared to the CD49 expression in an undifferentiated MSC population is indicative of the cell population being suitable as a therapeutic, wherein said differentiated MSC population and said undifferentiated MSC population are derived from the same donor.

According to some embodiments of the invention, the MSCs are derived from the bone marrow.

According to some embodiments of the invention, more than 95 % of the cells of said population of undifferentiated MSCs express CD73, CD90 and CD105.

According to some embodiments of the invention, the populations of undifferentiated MSCs do not express CD3, CD14, CD19, CD34, CD45, and HLA-DR as determined by flow cytometry.

According to some embodiments of the invention, the incubating is effected for no more than 6 days.

According to some embodiments of the invention, the number of cells in the cell population is at least 1 x 10^6 cells.

According to some embodiments of the invention, the number of cells being greater than 85 % is indicative of the cell population being suitable as a therapeutic.

According to some embodiments of the invention, the method further comprises determining the amount of neurotrophic factor secreted from the cells, wherein an amount of said neurotrophic factor being above a predetermined level is further indicative of the cell population being suitable as a therapeutic.

According to some embodiments of the invention, the neurotrophic factor is GDNF.

According to some embodiments of the invention, the neurotrophic factor is selected from the group consisting of GDNF, VEGF and HGF.

According to some embodiments of the invention, the predetermined level is at least 5 times greater than the amount of GDNF secreted from a non-differentiated mesenchymal stem cell obtained from the same donor.

According to some embodiments of the invention, the differentiating medium is devoid of a phosphodiesterase inhibitor.

According to some embodiments of the invention, the differentiating medium is devoid of triiodothyronine.

According to some embodiments of the invention, the phosphodiesterase inhibitor comprises IBMX.

According to some embodiments of the invention, the differentiating medium is devoid of xeno derived components.

According to some embodiments of the invention, the differentiating medium is devoid of antibiotics.

According to some embodiments of the invention, the method further comprises culturing said population of undifferentiated MSCs prior to said incubating, wherein said culturing is effected under conditions that do not promote cell differentiation.

According to some embodiments of the invention, the culturing is effected for three days following seeding of said undifferentiated MSCs.

According to some embodiments of the invention, the seeding is effected at a density of about 6000-8000 cm^2.

According to some embodiments of the invention, the culturing is effected in a culture medium comprising platelet lysate.

According to some embodiments of the invention, the percentage of said platelet lysate in said culture medium is about 10 %.

According to some embodiments of the invention, the culture medium further comprises L-glutamine, sodium pyruvate and heparin.

According to some embodiments of the invention, the analyzing is effected by flow cytometry.

According to some embodiments of the invention, at least 90 % of the cells of the population express CD49a.

According to some embodiments of the invention, at least 80 % of the cells of the population express CD49a.

According to some embodiments of the invention, the cells have been ex vivo differentiated by incubation in a differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP.

According to some embodiments of the invention, at least 90 % of the cells of the population express CD49a.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a representative flow cytometric analysis of CD49a expression on the surface of MSC (black) and MSC-NTF (heavy black) cells of the same donor at the end of differentiation. The dotted line to the left is the isotype control (MFI of the isotype control is 0.395, of MSC 2.83 and of MSC-NTF 13.5).

FIGs. 2A-B are graphs illustrating the amount of GDF-15 (Figure 2A) and IL-8 (Figure 2B) in ALS patient–derived bone marrow MSCs prior to and following differentiation.

FIGs. 3A-B are graphs illustrating the stability of the cells in storage medium. Cells were incubated in syringes at 2-8 °C. At 24, 48, 72 and 96 hours the cells were sampled and recovery of viable cells and viability were evaluated at each time point (Figure 3A). At each time point, cells were also seeded in culture medium for 3 days. The recovery of viable cells and viability were evaluated at the end of each of the 3 days' culture period (calculated as the % recovery of cells seeded at time 0; Figure 3B). Results are presented as average of 2 experiments.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to a method of qualifying neurotrophic factor secreting cells based on cell surface marker expression.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Neurotrophic factors (NTFs) are secreted proteins that regulate the survival, functional maintenance and phenotypic development of neuronal cells. Alterations in

NTF levels are involved in triggering programmed cell-death in neurons and thus contribute to the pathogenesis of Parkinson's disease and other neurodegenerative diseases.

However, the direct use of neurotrophic factors is not applicable as they do not pass the blood-brain barrier and do not distribute properly following systemic injection. Therefore, other strategies must be developed in order to take advantage of their therapeutic properties.

Protocols for differentiating human mesenchymal stem cells (MSCs) into neurotrophic factor secreting cells are known in the art – see for example WO 2006/134602 and WO 2009/144718.

The present inventors have previously developed a new one step differentiation protocol which enhances the secretion of neurotrophic factors from MSCs. The level of secretion of glial derived growth factor (GDNF) and brain derived neurotrophic factor (BDNF) was shown to be consistently up-regulated following the differentiation process, with GDNF being up-regulated by as much as 20 fold and BDNF by as much as three fold as compared to the corresponding non-differentiated cell population obtained from the same donor.

The protocol involves direct differentiation of undifferentiated MSCs in a single medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP.

The present inventors have now discovered a unique and simple way of selecting for mesenchymal stem cell populations which have been successfully differentiated according to this protocol based on expression of a cell surface marker. Of the myriad of potential cell surface markers expressed on these differentiated cells, the present inventors have found CD49a can be used as a single marker to substantiate successful differentiation.

As illustrated in Figure 1, following a successful differentiation, more than 80 % of the cells obtained expressed CD49a on their cell surface. In contrast, only about 65 % of the cells prior to differentiation expressed CD49a on their cell surface. In addition, the present inventors showed that the level of CD49a expression on a successfully differentiated MSC was higher than the level of CD49a expression on a non-differentiated MSC.

Thus, according to one aspect of the present invention there is provided a method of qualifying whether a cell population is a suitable therapeutic comprising:

(a) incubating a population of undifferentiated mesenchymal stem cells (MSCs) in a differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP for at least two days to obtain a population of differentiated MSCs; and

(b) analyzing the expression of CD49a in the differentiated MSC population, wherein an amount of CD49a above a predetermined level indicative of the cell population being suitable as a therapeutic.

As used herein, the phrase "suitable therapeutic" refers to the suitability of the cell population for treating neurodegenerative diseases and immune diseases (e.g. autoimmune diseases). According to a particular embodiment, cells which are suitable therapeutics are those that secrete sufficient neurotrophic factors that they are capable of having a therapeutic effect for a particular disease.

The term "neurodegenerative disease" is used herein to describe a disease which is caused by damage to the central nervous system. Exemplary neurodegenerative diseases which may be treated using the cells and methods according to the present invention include for example: Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease, Multiple System Atrophy (MSA), Huntington's disease, Alzheimer's disease, Rett Syndrome, lysosomal storage diseases ("white matter disease" or glial/demyelination disease, as described, for example by Folkerth, J. Neuropath. Exp. Neuro., September 1999, 58:9), including Sanfilippo, Gaucher disease, Tay Sachs disease (beta hexosaminidase deficiency), other genetic diseases, multiple sclerosis (MS), brain injury or trauma caused by ischemia, accidents, environmental insult, etc., spinal cord damage, ataxia. In addition, the present invention may be used to reduce and/or eliminate the effects on the central nervous system of a stroke in a patient, which is otherwise caused by lack of blood flow or ischemia to a site in the brain of the patient or which has occurred from physical injury to the brain and/or spinal cord. Neurodegenerative diseases also include neurodevelopmental disorders including for example, autism-spectrum disorders and related neurological diseases such as schizophrenia, among numerous others.

Autoimmune diseases of the nervous system which may be treated using the cells described herein include for example, multiple sclerosis and myasthenia gravis, Guillain bar syndrome, Multiple system Atrophy (MSA; a sporadic, progressive, adult-onset neurodegenerative disorder associated with varying degrees of parkinsonism, autonomic dysfunction and cerebellar ataxia). Other autoimmune diseases are described in Kraker et al., Curr Neuropharmacol. 2011 September; 9(3): 400–408.

The cells of the present invention show enhanced immunomodulatory effect as compared to non-differentiated bone marrow derived MSCs (see Table 5 herein below). Thus, the cells of the present invention may be useful in the treatment of any immune-related or inflammatory disorder.

As used herein the phrase "inflammatory disorders" includes but is not limited to chronic inflammatory diseases and acute inflammatory diseases. Examples of such diseases and conditions are summarized infra.

Inflammatory diseases associated with hypersensitivity

Examples of hypersensitivity include, but are not limited to, Type I hypersensitivity, Type II hypersensitivity, Type III hypersensitivity, Type IV hypersensitivity, immediate hypersensitivity, antibody mediated hypersensitivity, immune complex mediated hypersensitivity, T lymphocyte mediated hypersensitivity and DTH.

Type I or immediate hypersensitivity, such as asthma.

Type II hypersensitivity include, but are not limited to, rheumatoid diseases, rheumatoid autoimmune diseases, rheumatoid arthritis (Krenn V. *et al.,* Histol Histopathol 2000 Jul;15 (3):791), spondylitis, ankylosing spondylitis (Jan Voswinkel *et al.,* Arthritis Res 2001; 3 (3): 189), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Erikson J. *et al.,* Immunol Res 1998;17 (1-2):49), sclerosis, systemic sclerosis (Renaudineau Y. *et al.,* Clin Diagn Lab Immunol. 1999 Mar;6 (2):156); Chan OT. *et al.,* Immunol Rev 1999 Jun;169:107), glandular diseases, glandular autoimmune diseases, pancreatic autoimmune diseases, diabetes, Type I diabetes (Zimmet P. Diabetes Res Clin Pract 1996 Oct;34 Suppl:S125), thyroid diseases, autoimmune thyroid diseases, Graves' disease (Orgiazzi J. Endocrinol Metab Clin North Am 2000 Jun;29 (2):339), thyroiditis, spontaneous autoimmune thyroiditis

(Braley-Mullen H. and Yu S, J Immunol 2000 Dec 15;165 (12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.,* Nippon Rinsho 1999 Aug;57 (8):1810), myxedema, idiopathic myxedema (Mitsuma T. Nippon Rinsho. 1999 Aug;57 (8):1759); autoimmune reproductive diseases, ovarian diseases, ovarian autoimmunity (Garza KM. *et al.,* J Reprod Immunol 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.,* Am J Reprod Immunol. 2000 Mar;43 (3):134), repeated fetal loss (Tincani A. *et al.,* Lupus 1998;7 Suppl 2:S107-9), neurodegenerative diseases, neurological diseases, neurological autoimmune diseases, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.,* J Neural Transm Suppl. 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, Int Rev Immunol 1999;18 (1-2):83), motor neuropathies (Kornberg AJ. J Clin Neurosci. 2000 May;7 (3):191), Guillain-Barre syndrome, neuropathies and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000 Apr;319 (4):234), myasthenic diseases, Lambert-Eaton myasthenic syndrome (Takamori M. Am J Med Sci. 2000 Apr;319 (4):204), paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy, non-paraneoplastic stiff man syndrome, cerebellar atrophies, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette syndrome, polyendocrinopathies, autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol (Paris) 2000 Jan;156 (1):23); neuropathies, dysimmune neuropathies (Nobile-Orazio E. *et al.,* Electroencephalogr Clin Neurophysiol Suppl 1999;50:419); neuromyotonia, acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.,* Ann N Y Acad Sci. 1998 May 13;841:482), cardiovascular diseases, cardiovascular autoimmune diseases, atherosclerosis (Matsuura E. *et al.,* Lupus. 1998;7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998;7 Suppl 2:S132), thrombosis (Tincani A. *et al.,* Lupus 1998;7 Suppl 2:S107-9), granulomatosis, Wegener's granulomatosis, arteritis, Takayasu's arteritis and Kawasaki syndrome (Praprotnik S. *et al.,* Wien Klin Wochenschr 2000 Aug 25;112 (15-16):660); anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.,* Semin Thromb Hemost.2000;26 (2):157); vasculitises, necrotizing small vessel vasculitises, microscopic polyangiitis, Churg and Strauss syndrome, glomerulonephritis, pauci-immune focal necrotizing glomerulonephritis, crescentic glomerulonephritis (Noel LH. Ann Med Interne (Paris).

2000 May;151 (3):178); antiphospholipid syndrome (Flamholz R. *et al.,* J Clin Apheresis 1999;14 (4):171); heart failure, agonist-like beta-adrenoceptor antibodies in heart failure (Wallukat G. *et al.,* Am J Cardiol. 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999 Apr-Jun;14 (2):114); hemolytic anemia, autoimmune hemolytic anemia (Efremov DG. *et al.,* Leuk Lymphoma 1998 Jan;28 (3-4):285), gastrointestinal diseases, autoimmune diseases of the gastrointestinal tract, intestinal diseases, chronic inflammatory intestinal disease (Garcia Herola A. *et al.,* Gastroenterol Hepatol. 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16;138 (2):122), autoimmune diseases of the musculature, myositis, autoimmune myositis, Sjogren's syndrome (Feist E. *et al.,* Int Arch Allergy Immunol 2000 Sep;123 (1):92); smooth muscle autoimmune disease (Zauli D. *et al.,* Biomed Pharmacother 1999 Jun;53 (5-6):234), hepatic diseases, hepatic autoimmune diseases, autoimmune hepatitis (Manns MP. J Hepatol 2000 Aug;33 (2):326) and primary biliary cirrhosis (Strassburg CP. *et al.,* Eur J Gastroenterol Hepatol. 1999 Jun;11 (6):595).

Type IV or T cell mediated hypersensitivity, include, but are not limited to, rheumatoid diseases, rheumatoid arthritis (Tisch R, McDevitt HO. Proc Natl Acad Sci U S A 1994 Jan 18;91 (2):437), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Datta SK., Lupus 1998;7 (9):591), glandular diseases, glandular autoimmune diseases, pancreatic diseases, pancreatic autoimmune diseases, Type 1 diabetes (Castano L. and Eisenbarth GS. Ann. Rev. Immunol. 8:647); thyroid diseases, autoimmune thyroid diseases, Graves' disease (Sakata S. *et al.,* Mol Cell Endocrinol 1993 Mar;92 (1):77); ovarian diseases (Garza KM. *et al.,* J Reprod Immunol 1998 Feb;37 (2):87), prostatitis, autoimmune prostatitis (Alexander RB. *et al.,* Urology 1997 Dec;50 (6):893), polyglandular syndrome, autoimmune polyglandular syndrome, Type I autoimmune polyglandular syndrome (Hara T. *et al.,* Blood. 1991 Mar 1;77 (5):1127), neurological diseases, autoimmune neurological diseases, multiple sclerosis, neuritis, optic neuritis (Soderstrom M. *et al.,* J Neurol Neurosurg Psychiatry 1994 May;57 (5):544), myasthenia gravis (Oshima M. *et al.,* Eur J Immunol 1990 Dec;20 (12):2563), stiff-man syndrome (Hiemstra HS. *et al.,* Proc Natl Acad Sci U S A 2001 Mar 27;98 (7):3988), cardiovascular diseases, cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.,* J Clin Invest 1996 Oct 15;98 (8):1709), autoimmune

thrombocytopenic purpura (Semple JW. *et al.,* Blood 1996 May 15;87 (10):4245), anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.,* Viral Immunol 1998;11 (1):9), hemolytic anemia (Sallah S. *et al.,* Ann Hematol 1997 Mar;74 (3):139), hepatic diseases, hepatic autoimmune diseases, hepatitis, chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), biliary cirrhosis, primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551), nephric diseases, nephric autoimmune diseases, nephritis, interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140), connective tissue diseases, ear diseases, autoimmune connective tissue diseases, autoimmune ear disease (Yoo TJ. *et al.*, Cell Immunol 1994 Aug;157 (1):249), disease of the inner ear (Gloddek B. *et al.*, Ann N Y Acad Sci 1997 Dec 29;830:266), skin diseases, cutaneous diseases, dermal diseases, bullous skin diseases, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of delayed type hypersensitivity include, but are not limited to, contact dermatitis and drug eruption.

Examples of types of T lymphocyte mediating hypersensitivity include, but are not limited to, helper T lymphocytes and cytotoxic T lymphocytes.

Examples of helper T lymphocyte-mediated hypersensitivity include, but are not limited to, T_h1 lymphocyte mediated hypersensitivity and T_h2 lymphocyte mediated hypersensitivity.

Autoimmune diseases

Include, but are not limited to, cardiovascular diseases, rheumatoid diseases, glandular diseases, gastrointestinal diseases, cutaneous diseases, hepatic diseases, neurological diseases, muscular diseases, nephric diseases, diseases related to reproduction, connective tissue diseases and systemic diseases.

Examples of autoimmune cardiovascular diseases include, but are not limited to atherosclerosis (Matsuura E. *et al.,* Lupus. 1998;7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998;7 Suppl 2:S132), thrombosis (Tincani A. *et al.,* Lupus 1998;7 Suppl 2:S107-9), Wegener's granulomatosis, Takayasu's arteritis, Kawasaki syndrome (Praprotnik S. *et al.,* Wien Klin Wochenschr 2000 Aug 25;112 (15-16):660), anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.,* Semin Thromb Hemost.2000;26 (2):157), necrotizing small vessel vasculitis, microscopic polyangiitis, Churg and Strauss syndrome, pauci-immune focal necrotizing and crescentic glomerulonephritis

(Noel LH. Ann Med Interne (Paris). 2000 May;151 (3):178), antiphospholipid syndrome (Flamholz R. *et al.,* J Clin Apheresis 1999;14 (4):171), antibody-induced heart failure (Wallukat G. *et al.,* Am J Cardiol. 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999 Apr-Jun;14 (2):114; Semple JW. *et al.,* Blood 1996 May 15;87 (10):4245), autoimmune hemolytic anemia (Efremov DG. *et al.,* Leuk Lymphoma 1998 Jan;28 (3-4):285; Sallah S. *et al.,* Ann Hematol 1997 Mar;74 (3):139), cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.,* J Clin Invest 1996 Oct 15;98 (8):1709) and anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.,* Viral Immunol 1998;11 (1):9).

Examples of autoimmune rheumatoid diseases include, but are not limited to rheumatoid arthritis (Krenn V. *et al.,* Histol Histopathol 2000 Jul;15 (3):791; Tisch R, McDevitt HO. Proc Natl Acad Sci units S A 1994 Jan 18;91 (2):437) and ankylosing spondylitis (Jan Voswinkel *et al.,* Arthritis Res 2001; 3 (3): 189).

Examples of autoimmune glandular diseases include, but are not limited to, pancreatic disease, Type I diabetes, thyroid disease, Graves' disease, thyroiditis, spontaneous autoimmune thyroiditis, Hashimoto's thyroiditis, idiopathic myxedema, ovarian autoimmunity, autoimmune anti-sperm infertility, autoimmune prostatitis and Type I autoimmune polyglandular syndrome. Diseases include, but are not limited to autoimmune diseases of the pancreas, Type 1 diabetes (Castano L. and Eisenbarth GS. Ann. Rev. Immunol. 8:647; Zimmet P. Diabetes Res Clin Pract 1996 Oct;34 Suppl:S125), autoimmune thyroid diseases, Graves' disease (Orgiazzi J. Endocrinol Metab Clin North Am 2000 Jun;29 (2):339; Sakata S. *et al.,* Mol Cell Endocrinol 1993 Mar;92 (1):77), spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, J Immunol 2000 Dec 15;165 (12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.,* Nippon Rinsho 1999 Aug;57 (8):1810), idiopathic myxedema (Mitsuma T. Nippon Rinsho. 1999 Aug;57 (8):1759), ovarian autoimmunity (Garza KM. *et al.,* J Reprod Immunol 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.,* Am J Reprod Immunol. 2000 Mar;43 (3):134), autoimmune prostatitis (Alexander RB. *et al.,* Urology 1997 Dec;50 (6):893) and Type I autoimmune polyglandular syndrome (Hara T. *et al.,* Blood. 1991 Mar 1;77 (5):1127).

Examples of autoimmune gastrointestinal diseases include, but are not limited to, chronic inflammatory intestinal diseases (Garcia Herola A. *et al.,* Gastroenterol

Hepatol. 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16;138 (2):122), colitis, ileitis and Crohn's disease.

Examples of autoimmune cutaneous diseases include, but are not limited to, autoimmune bullous skin diseases, such as, but are not limited to, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of autoimmune hepatic diseases include, but are not limited to, hepatitis, autoimmune chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551; Strassburg CP. *et al.,* Eur J Gastroenterol Hepatol. 1999 Jun;11 (6):595) and autoimmune hepatitis (Manns MP. J Hepatol 2000 Aug;33 (2):326).

Examples of autoimmune neurological diseases include, but are not limited to, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.,* J Neural Transm Suppl. 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, Int Rev Immunol 1999;18 (1-2):83; Oshima M. *et al.,* Eur J Immunol 1990 Dec;20 (12):2563), neuropathies, motor neuropathies (Kornberg AJ. J Clin Neurosci. 2000 May;7 (3):191); Guillain-Barre syndrome and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000 Apr;319 (4):234), myasthenia, Lambert-Eaton myasthenic syndrome (Takamori M. Am J Med Sci. 2000 Apr;319 (4):204); paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy and stiff-man syndrome (Hiemstra HS. *et al.,* Proc Natl Acad Sci units S A 2001 Mar 27;98 (7):3988); non-paraneoplastic stiff man syndrome, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette syndrome and autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol (Paris) 2000 Jan;156 (1):23); dysimmune neuropathies (Nobile-Orazio E. *et al.,* Electroencephalogr Clin Neurophysiol Suppl 1999;50:419); acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.,* Ann N Y Acad Sci. 1998 May 13;841:482), neuritis, optic neuritis (Soderstrom M. *et al.,* J Neurol Neurosurg Psychiatry 1994 May;57 (5):544) and neurodegenerative diseases.

Examples of autoimmune muscular diseases include, but are not limited to, myositis, autoimmune myositis and primary Sjogren's syndrome (Feist E. *et al.,* Int

Arch Allergy Immunol 2000 Sep;123 (1):92) and smooth muscle autoimmune disease (Zauli D. *et al.,* Biomed Pharmacother 1999 Jun;53 (5-6):234).

Examples of autoimmune nephric diseases include, but are not limited to, nephritis and autoimmune interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140).

Examples of autoimmune diseases related to reproduction include, but are not limited to, repeated fetal loss (Tincani A. *et al.,* Lupus 1998;7 Suppl 2:S107-9).

Examples of autoimmune connective tissue diseases include, but are not limited to, ear diseases, autoimmune ear diseases (Yoo TJ. *et al.*, Cell Immunol 1994 Aug;157 (1):249) and autoimmune diseases of the inner ear (Gloddek B. *et al.*, Ann N Y Acad Sci 1997 Dec 29;830:266).

Examples of autoimmune systemic diseases include, but are not limited to, systemic lupus erythematosus (Erikson J. *et al.,* Immunol Res 1998;17 (1-2):49) and systemic sclerosis (Renaudineau Y. *et al.,* Clin Diagn Lab Immunol. 1999 Mar;6 (2):156); Chan OT. *et al.,* Immunol Rev 1999 Jun;169:107).

Infectious diseases

Examples of infectious diseases include, but are not limited to, chronic infectious diseases, subacute infectious diseases, acute infectious diseases, viral diseases, bacterial diseases, protozoan diseases, parasitic diseases, fungal diseases, mycoplasma diseases and prion diseases.

Graft rejection diseases

Examples of diseases associated with transplantation of a graft include, but are not limited to, graft rejection, chronic graft rejection, subacute graft rejection, hyperacute graft rejection, acute graft rejection and graft versus host disease.

Allergic diseases

Examples of allergic diseases include, but are not limited to, asthma, hives, urticaria, pollen allergy, dust mite allergy, venom allergy, cosmetics allergy, latex allergy, chemical allergy, drug allergy, insect bite allergy, animal dander allergy, stinging plant allergy, poison ivy allergy and food allergy.

Cancerous diseases

Examples of cancer include but are not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia. Particular examples of cancerous diseases but are not

limited to: Myeloid leukemia such as Chronic myelogenous leukemia. Acute myelogenous leukemia with maturation. Acute promyelocytic leukemia, Acute nonlymphocytic leukemia with increased basophils, Acute monocytic leukemia. Acute myelomonocytic leukemia with eosinophilia; Malignant lymphoma, such as Birkitt's Non-Hodgkin's; Lymphoctyic leukemia, such as Acute lumphoblastic leukemia. Chronic lymphocytic leukemia; Myeloproliferative diseases, such as Solid tumors Benign Meningioma, Mixed tumors of salivary gland, Colonic adenomas; Adenocarcinomas, such as Small cell lung cancer, Kidney, Uterus, Prostate, Bladder, Ovary, Colon, Sarcomas, Liposarcoma, myxoid, Synovial sarcoma, Rhabdomyosarcoma (alveolar), Extraskeletel myxoid chonodrosarcoma, Ewing's tumor; other include Testicular and ovarian dysgerminoma, Retinoblastoma, Wilms' tumor, Neuroblastoma, Malignant melanoma, Mesothelioma, breast, skin, prostate, and ovarian.

According to a particular embodiment, the method described herein is for qualifying whether the cell populations are suitable for treating ALS.

The term "mesenchymal stem cell" or "MSC" is used interchangeably for adult cells which are not terminally differentiated, which can divide to yield cells that are either stem cells, or which, irreversibly differentiate to give rise to cells of a mesenchymal (chrondocyte, osteocyte and adipocyte) cell lineage. The mesenchymal stem cells of the present invention, in at least some embodiments, may be of an autologous (e.g. syngeneic) or allogeneic source.

Populations of MSCs typically express particular markers on their cell surface. According to a particular embodiment, the undifferentiated MSCs express CD105, CD73 and CD90 on the cell surface (e.g. >95% positive) and lack expression (e.g. < 2% positive) of CD3, CD14, CD19, CD34, CD45, and HLA-DR as determined by flow cytometry.

Exemplary antibodies that may be used to verify the presence of mesenchymal stem cells include CD44 FITC conjugated, BD Biosciences, CD73 PE conjugated (BD Pharmingen), CD73 PE conjugated, BD Biosciences, CD90 PE-Cy5 conjugated (eBioscience) CD90 PE conjugated, BD Biosciences CD105 PE conjugated (Beckman Coulter) CD3 PerCP conjugated, BD Biosciences, CD14 FITC conjugated (eBioscience) CD14 FITC conjugated, BD Biosciences CD19 PE-Cy5 conjugated

(eBioscience) CD19 FITC conjugated, BD Biosciences CD34 FITC conjugated BD Biosciences (Beckman Coulter), CD45 PE conjugated (eBioscience) CD45 PerCP conjugated, BD Biosciences and HLA-DR PE-Cy5 conjugated (BD Pharmingen). HLA-DR PerCP conjugated, BD Biosciences.

Another method for verifying the presence of mesenchymal stem cells is by showing that the cells are capable of differentiating into multi-lineages such as for example adipocytes, osteocytes and chondrocytes. This may be effected for example using Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems).

According to a preferred embodiment of this aspect of the present invention the mesenchymal stem cells are not genetically manipulated (i.e. transformed with an expression construct) to generate the cells and cell populations described herein.

It will be appreciated that the cells of the present invention, in at least some embodiments, may be derived from any stem cell, although preferably not embryonic stem (ES) cells.

Mesenchymal stem cells may be isolated from various tissues including but not limited to bone marrow, peripheral blood, blood, placenta and adipose tissue. A method of isolating mesenchymal stem cells from peripheral blood is described by Kassis et al [Bone Marrow Transplant. 2006 May; 37(10):967-76]. A method of isolating mesenchymal stem cells from placental tissue is described by Brooke G et al. [Br J Haematol. 2009 Feb; 144 (4):571-9].

Methods of isolating and culturing adipose tissue, placental and cord blood mesenchymal stem cells are described by Kern et al [Stem Cells, 2006; 24:1294-1301].

According to a preferred embodiment of this aspect of the present invention, the mesenchymal stem cells are human.

Bone marrow can be isolated from the iliac crest or the sternum of an individual by aspiration. Low-density BM mononuclear cells (BMMNC) may be separated by FICOLL®-PAQUE density gradient centrifugation. In order to obtain mesenchymal stem cells, a cell population comprising the mesenchymal stem cells (e.g. BMMNC) may be cultured in a proliferating medium capable of maintaining and/or expanding the cells in the presence of platelet lysate. According to one embodiment the populations are plated on plastic surfaces (e.g. in a flask) and mesenchymal stem cells are isolated by

removing non-adherent cells. Alternatively mesenchymal stem cell may be isolated by FACS using mesenchymal stem cell markers.

Following isolation the cells may be expanded by culturing in a proliferation medium capable of maintaining and/or expanding the isolated cells *ex vivo* in the presence of platelet lysate. The proliferation medium may be DMEM, alpha-MEM or DMEM/F12. Typically, the glucose concentration in the medium is about 0.5 – 3 grams/litre.

The culturing may be effected on any suitable surface including plastic dishes and bioreactors suitable for culturing mesenchymal stem cells.

Platelet lysate may be prepared using any method known in the art. Platelet Rich Plasma (PRP) may be derived from blood bank donations determined free of infectious agents (i.e. HIV, HTLV, HCV, HBsAg). PRP containing bags may be stored at -80 ^{0}C and thawed in a 37 °C water bath. After thawing, the Platelet Rich Plasma is typically centrifuged to remove platelet particles and membranes. The Platelet lysate supernatant may then be collected and frozen at -80 °C until use. The Platelet lysate is tested for Endotoxin, Haemoglobin, pH, Total protein, Albumin, Osmolality Sterility and Mycoplasma.

The proliferation medium may comprise additional components, including for example L-glutamine, sodium pyruvate and heparin.

It will be appreciated that preferably when the mesenchymal stem cells are human, the platelet lysate is also obtained from human cells.

According to one embodiment, the proliferation/growth medium is devoid of xeno contaminants i.e. free of animal derived components such as serum, animal derived growth factors and albumin. Thus, according to this embodiment, the culturing is performed in the absence of xeno contaminants.

An exemplary mesenchymal stem cell isolation and propagation protocol is presented in the Examples section, herein below.

As mentioned, following propagation of mesenchymal stem cells in a platelet lysate containing medium, when an adequate number of undifferentiated cells are obtained, the cells are differentiated in a differentiating medium to generating cells useful for treating diseases.

According to a particular embodiment, the cells are reseeded in a fresh proliferation/growth medium (e.g. at a density of about 6000-8000 cells per cm^2) for 1 day, 2 days, 3 days, 4 days or 5 days prior to addition of the differentiation medium.

The phrase "undifferentiated MSCs" refers to MSCs that have not been cultured in a medium that induces differentiation. Thus, according to at least some embodiments of the present invention, following optional proliferation, the MSCs are contacted directly with the differentiation medium without any intervening pre-differentiation steps.

For differentiation, the undifferentiated MSCs of the present invention, in at least some embodiments are incubated in a medium comprising fibroblast growth factor (FGF), platelet derived growth factor (PDGF), heregulin and c-AMP. According to this embodiment each of fibroblast growth factor (FGF), platelet derived growth factor (PDGF), heregulin and c-AMP are mixed in a single medium and the culturing is effected in a single step.

According to one embodiment, the undifferentiated MSCs of the present invention are not pre-incubated in the presence of epidermal growth factor (EGF) and/or N2 supplement prior to this step and following the expansion step.

An exemplary concentration of bFGF which is contemplated for the differentiation medium of embodiments of this invention is optionally between 5-50 ng/ml, optionally between 10-40 ng/ml, optionally between 10-25 ng/ml.

An exemplary concentration of PDGF-AA which is contemplated for the differentiation medium of embodiments of this invention is optionally between 1-30 ng/ml, optionally between 1-20 ng/ml, optionally between 1-10 ng/ml, optionally between 2.5-10 ng/ml.

An exemplary concentration of heregulin β1 which is contemplated for the differentiation medium of embodiments of this invention is optionally between 5-100 ng/ml, 10-90 ng/ml, optionally between 25-75 ng/ml and optionally between 40-60 ng/ml.

An exemplary concentration of dbc-AMP which is contemplated for the differentiation medium of embodiments of this invention is optionally between 0.5-10 mM, optionally between 0.5-5mM and optionally between 0.5 and 2.5 mM.

According to one embodiment, the differentiating medium of this aspect of the present invention is devoid of a phosphodiesterase inhibitor (e.g. IBMX) i.e. the culturing is performed in the absence of a phosphodiesterase inhibitor.

According to another embodiment, the differentiating medium of this aspect of the present invention is devoid of triiodothyronine i.e. the culturing is performed in the absence of triiodothyronine.

Optionally, any of these embodiments and subembodiments may be combined, so that for example the differentiating medium may optionally be devoid of both a phosphodiesterase inhibitor and triiodothyronine.

Preferably, the MSCs are differentiated in the above described differentiating medium for at least one day, at least two days or at least 3 days. Preferably, the differentiating stage is not performed for more than five days.

The differentiating media used according to this aspect of the present invention are preferably xeno-free (devoid of serum) and devoid of any antibiotics i.e. the culturing is performed in the absence of xeno-contaminants.

Harvesting of the cells is typically carried out in an appropriate medium e.g. Hanks balanced salt solution (HBSS), Dulbecco Modified Eagle Medium (DMEM) RPMI, PBS etc. Hypothermic storage mediums are also contemplated (e.g. Hypothermosol).

Following the differentiation process, the cells obtained are analyzed for the expression of CD49a, wherein an amount of CD49a above a predetermined level indicative of the cell population being suitable as the therapeutic.

It will be appreciated that not all the cells obtained need to be analyzed for CD49a expression, but rather a sample thereof which provides information as to the state of the rest of the cell population.

Typically, the number of the cells in the sample is about 0.5 x 10^6 cells.

The number of cells obtained from a single donor is generally between 20 x 10^6 cells - 100 x 10^7 cells. Thus the number of cells may be about 20 x 10^6 cells, about 100 x 10^6 cells, about 200 x 10^6 cells are differentiated, about 300 x 10^6 cells, about 400 x 10^6 cells are differentiated, about 500 x 10^6 cells are differentiated, about 600 x 10^6 cells are differentiated, about 700 x 10^6 cells, about 800 x 10^6 cells, about 900 x 10^6 cells or about 100 x 10^7 cells.

As used herein, the term "CD49a" (also referred to as Integrin alpha 1) refers to the alpha 1 subunit of integrin receptor cell surface membrane protein that binds to the extracellular matrix. This protein heterodimerizes with the beta 1 subunit to form a cell-surface receptor for collagen and laminin. The heterodimeric receptor is involved in cell-cell adhesion.

The human protein sequence of CD49a is set forth in Uniprot No. P56199, NP_852478 and its mRNA sequence is set forth in NM_181501.

It will be appreciated that since CD49a forms a heterodimer on the surface of cells together with the CD49 beta1, the method of the present invention can also be effected by determining the amount of CD49beta1 on the surface of the differentiated cells.

Methods for analyzing expression of CD49a or CD49beta1 typically involve the use of antibodies which specifically recognize the antigen. Commercially available antibodies that recognize CD49a include for example those manufactured by R and D systems, Santa Cruz (Cat# SC-81733PE) or Biolegend (e.g. catalogue number 328303). The analyzing may be carried out using any method known in the art including flow cytometry, Western Blot, HPLC, in situ-PCR immunocytochemistry, mass spectrometry, radioimmunoassay, etc. According to a particular embodiment, the analyzing is effected using an antibody which specifically recognizes the protein.

For flow cytometry, the CD49a or CD49b1 antibody is attached to a fluorescent moiety and analyzed using a fluorescence-activated cell sorter (FACS).

As used herein, the term "flow cytometry" refers to an assay in which the proportion of a material (e.g. mesenchymal stem cells comprising a particular marker) in a sample is determined by labeling the material (e.g., by binding a labeled antibody to the material), causing a fluid stream containing the material to pass through a beam of light, separating the light emitted from the sample into constituent wavelengths by a series of filters and mirrors, and detecting the light.

A multitude of flow cytometers are commercially available including for e.g. Becton Dickinson FACScan and FACScalibur (BD Biosciences, Mountain View, CA). Antibodies that may be used for FACS analysis are taught in Schlossman S, Boumell L, et al, [Leucocyte Typing V. New York: Oxford University Press; 1995] and are widely commercially available.

For some methods, including flow cytometry, the cell populations need to be removed from the culture plate. Examples of agents that may be used to disperse the cells include, but are not limited to collagenase, dispase, accutase, trypsin (e.g. trypsin-EDTA, non-animal substitutes of trypsin such as TrypLE^{TM}), papain. Alternatively, or additionally trituration may also be performed to increase the dispersal of the cells.

An exemplary concentration of trypsin that may be used is 0.005-0.5 % trypsin-EDTA. The cells may be incubated with the dispersing agent for about 5-30 minutes, at a temperature of about 37 °C.

The cells are typically resuspended in a suitable medium including for example phosphate buffered saline (PBS), Hanks balanced salt solution (HBSS), Dulbecco Modified Eagle Medium (DMEM) RPMI, PBS etc.

In order to qualify that the cells are useful as a therapeutic, the amount of CD49a should be increased above a statistically significant level as compared to non-differentiated MSCs of the same donor and from the same organ.

According to a particular embodiment, in order to qualify that the cells are useful as a therapeutic, at least 80 % of the cells of the population should express CD49a, more preferably at least 85 % of the cells of the population should express CD49a, more preferably at least 90 % of the cells of the population should express CD49a, more preferably at least 95 % of the cells of the population should express CD49a.

According to another embodiment, in order to qualify that the cells are useful as a therapeutic, the level of CD49a expression (e.g. the mean fluorescent intensity) should be increased by at least two fold, more preferably at least 3 fold, more preferably at least 4 fold and even more preferably by at least 5 fold as compared to non-differentiated MSCs of the same donor and from the same organ.

It will be appreciated that using a flow cytometer, cell populations may be obtained which are more than 80 % positive for CD49a. Thus, for example, cell populations may be obtained which are 85 % positive for CD49a, 90 % positive for CD49a, 91 % positive for CD49a, 92 % positive for CD49a, 93 % positive for CD49a, 94 % positive for CD49a, 95 % positive for CD49a, 96 % positive for CD49a, 97 % positive for CD49a, 98 % positive for CD49a, 99 % positive for CD49a and even 100 % positive for CD49a.

The cells may be analyzed for expression of additional cell surface markers such as CD44. Cells which have a decrease in expression by at least 1.5 or at least 2 fold or more of CD44 may be qualified as being useful as a therapeutic.

The cells may be qualified or characterized in additional ways including for example karyotype analysis, morphology, cell number and viability, gram staining and sterility.

In addition, the cells may be analyzed for their level of neurotrophic factor (NTF) secretion.

For analysis of secreted NTFs, supernatant is collected from cultures of MSCs or of NTF-secreting cells at the end of the differentiation procedure described above, and cells are harvested and counted. The amount of NTFs such as Glial Derived Neurotrophic Factor, (GDNF) or Brain Derived Neurotrophic Factor (BDNF) in the cell's culture supernatants may be quantified by using a GDNF or BDNF ELISA assay (GDNF DuoSet DY212; BDNF DuoSet DY248; R&D Systems) according to the manufacturer's protocol, for example and without limitation. The amount of IGF-1 can be quantified using an IGF ELISA assay (IGF-1 DuoSet Cat No. DY291; R&D System), for example and without limitation.

The amount of VEGF can be quantified using a VEGF ELISA assay (VEGF DuoSet R&D systems, Cat: DY293B) for example and without limitation. The amount of HGF can be quantified using an HGF ELISA assay (HGF DuoSet R&D systems, Cat: DY294) for example and without limitation.

Preferably, the amount of GDNF secreted by the cells of the present invention is increased by at least 2 fold, at least 3 fold, at least 4 fold, at least 5 fold, at least 6 fold, at least 7 fold, at least 8 fold the secretion of the same population of mesenchymal stem cells without differentiation.

The specific productivity of GDNF is from about 200-2000 pg/10^6 cells.

According to one embodiment, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 % or more of a population of the differentiated cells of the present invention secrete BDNF.

Preferably, the amount of BDNF secreted by the cells of the present invention is increased by at least 1.5 fold, at least 2 fold, at least 2.5 fold, at least 3 fold the secretion of the same population of mesenchymal stem cells without differentiation.

The specific productivity of BDNF is from about 500-8000 pg/10^6 cells.

The cells of the present invention differ from non-differentiated bone marrow derived mesenchymal stem cells in a variety of different ways.

Thus, for example, the cells of the present invention secrete at least 5 fold more GDF-15 than non-differentiated MSCs as measured by an ELISA assay for GDF-15 (e.g. R&D Systems, Cat # DY957 or equivalent).

Furthermore, the cells of the present invention secrete at least 10 fold, 20 fold or even 30 fold more IL-8 than non-differentiated MSCs as measured by an ELISA assay for IL-8 (e.g. R&D Systems, Cat # DY208-05 or equivalent).

In addition, the cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold the amount of any one of the polypeptides 1-82, listed in Table 2. According to another embodiment, the cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold the amount of each of the polypeptides 1-82, listed in Table 2. The cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold less of at least one of the polypeptides 83-122, listed in Table 2. The cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold less of each of the polypeptides 83-122, listed in Table 2.

The cells of the present invention may be distinguished from non-differentiated MSCs according to expression of particular genes. This may be measured by analyzing the amount of mRNA there is present in the cells encoded by the gene.

The cells of the present invention express at least 6 fold, 8 fold or even 10 fold the amount of any one of the genes 1-41, listed in Table 3. According to another embodiment, the cells of the present invention express at least 6 fold, 8 fold, 10 fold, 20 fold, or even 30 fold the amount of each of the genes 1-41, listed in Table 3. The cells of the present invention express at least 6 fold, 8 fold, 10 fold, 20 fold or even 30 fold less of at least one of the genes 42-56, listed in Table 3. The cells of the present invention express at least 6 fold, 8 fold, 10 fold or even 20 fold less of each of the genes 42-56, listed in Table 3.

The cells of the present invention differ from other bone marrow mesenchymal stem cell-derived NTF secreting cells (e.g. those disclosed in WO2009/144718 - those cells are referred to herein as 2 step protocol NTFs).

Thus the cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold the amount of any one of the polypeptides 1 and/or 9, listed in Table 4 as compared to 2 step protocol NTFs. The cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold less of at least one of the polypeptides 2-8, listed in Table 4 as compared to 2 step protocol NTFs. The cells of the present invention comprise at least 2 fold, 4 fold, 6 fold, 8 fold or even 10 fold less of each of the polypeptides 2-8, listed in Table 4 as compared to 2 step protocol NTFs.

The cells of the present invention express at least 2 fold, 4 fold or even 6 fold the amount of any one of the genes 1-82, listed in Table 5, as compared to 2 step protocol NTFs. According to another embodiment, the cells of the present invention express at least 2 fold, 4 fold or even 6 fold the amount of each of the genes 1-82 listed in Table 5 as compared to 2 step protocol NTFs. The cells of the present invention express at least 2 fold, 4 fold or even 6 fold less of at least one of the genes 83-126, listed in Table 5 as compared to 2 step protocol NTFs. The cells of the present invention express at least 2 fold, 4 fold or even 6 fold less of each of the genes 83-126, listed in Table 5 as compared to 2 step protocol NTFs.

Other distinguishing features of the cells of the present invention are provided in WO 2014/024183.

Once qualified, the cells may be labeled accordingly and preserved according to methods known in the art (e.g. frozen or cryopreserved) or may be directly administered to the subject.

As mentioned, the cells of this aspect of the present invention may be useful in treating immune or inflammatory related diseases.

Thus, according to another aspect of the present invention there is provided a method of treating an immune or inflammatory related disease in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of mesenchymal stem cells which have been ex vivo differentiated to secrete neurotrophic factors, thereby treating the disease.

Examples of such diseases have been provided herein above.

According to a particular embodiment, the immune or inflammatory related disease is not a neurodegenerative disease.

According to another embodiment, the immune or inflammatory related disease is not an immune disease of the nervous system.

According to still another embodiment, the immune or inflammatory related disease is not myasthenia gravis.

Methods of obtaining mesenchymal stem cells which have been ex vivo differentiated to secrete neurotrophic factors are disclosed in WO 2014/024183, WO2006/134602 and WO2009/144718.

The cells can be administered either *per se* or, preferably as a part of a pharmaceutical composition that further comprises a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the chemical conjugates described herein, with other chemical components such as pharmaceutically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to a subject.

Hereinafter, the term "pharmaceutically acceptable carrier" refers to a carrier or a diluent that does not cause significant irritation to a subject and does not abrogate the biological activity and properties of the administered compound. Examples, without limitations, of carriers are propylene glycol; saline; emulsions; buffers; culture medium such as DMEM or RPMI; hypothermic storage medium containing components that scavenge free radicals, provide pH buffering, oncotic/osmotic support, energy substrates and ionic concentrations that balance the intracellular state at low temperatures; and mixtures of organic solvents with water.

Typically, the pharmaceutical carrier preserves the number of cells (e.g. is not reduced by more than 90 %) in the composition for at least 24 hours, at least 48 hours or even at least 96 hours.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound and maintain cells viability at a pre-determined temperature for a suitable period of time before transplantation/injection. Examples, without limitation, of excipients include albumin, plasma, serum and cerebrospinal fluid (CSF), antioxidants such as N-Acetylcysteine (NAC) or resveratrol.

According to a preferred embodiment of the present invention, the pharmaceutical carrier is an aqueous solution of buffer or a culture medium such as DMEM.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. Preferably, a dose is formulated in an animal model to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals.

The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. Further information may be obtained from clinical studies – see for example Salem HK et al., Stem Cells 2010; 28:585-96; and Uccelli et al. Lancet Neurol. 2011; 10:649-56).

The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition, (see e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer and additional agents as described herein above.

Dosage amount and interval may be adjusted individually to levels of the active ingredient which are sufficient to effectively cause an immunomodulatory effect. Dosages necessary to achieve the desired effect will depend on individual characteristics and route of administration.

Depending on the severity and responsiveness of the condition to be treated, dosing of cells can be of a single or a plurality of administrations, with course of

treatment lasting from several days to several weeks or months depending when diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the individual being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual changing condition.

The cells of the present invention, in at least some embodiments, may be prepackaged in unit dosage forms in a syringe ready for use. The syringe may be labeled with the name of the cells and their source. The labeling may also comprise information related to the function of the cells (e.g. the amount of neurotrophic factor secreted therefrom). The syringe may be packaged in a packaging which is also labeled with information regarding the cells.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as

individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons,

Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996).

Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader.

EXAMPLE 1

Analysis of surface markers in differentiated cells

MATERIALS AND METHODS

Bone marrow aspiration (BMA): Fresh bone marrow was aspirated according to the routine Medical Center procedure from the patient's iliac-crest under local

anesthesia and sedation by an anesthetist. Bone marrow (30-60 ml) was aspirated using aspiration needles into heparin containing tubes.

Separation of MNC and enrichment of MSC: This step involves separation of mononuclear cells (MNC) from total bone marrow.

The Human Multipotent Mesenchymal stromal cells (MSC), estimated to comprise 0.01% of total bone marrow MNC, are enriched *in-vitro* from MNC, by virtue of their ability to adhere to plastic.

Bone marrow aspirate was diluted 1:1 (v:v) in Hank's Balanced Salt Solution (HBSS), and MNC were separated from total bone marrow cells by Ficoll density gradient centrifugation.

MNC were counted and cell number and viability were determined by the Trypan Blue dye exclusion test. The yield of MNC recovered after density gradient centrifugation varied between donors and depends on the volume of bone marrow collected. The yield of MNC recovered from 30-50 ml of bone marrow aspirate of ALS patients ranged between 70-400 x 10^6 MNC and was sufficient for isolating the number of MSC necessary for the entire production process.

The medium used for seeding the primary bone marrow mononuclear cells and propagating the MSCs throughout the production process was designated Platelets Growth medium (PM). The PM medium was used throughout the MSC production process (Passage 0 - Passage 4) [P0-P4] and contained low glucose DMEM, L-Glutamine sodium pyruvate, heparin and platelet lysate.

MNCs were seeded at a density of 100,000-400,000 cells/cm^2 in flasks in PM/flask and incubated overnight in a 37 °C/5% CO_2 humidified incubator. The next day, the cell culture was examined under the microscope. At this stage, non-adherent, mononuclear cell were floating in the culture supernatant and plastic-adherent MSC were attached to the flask surface. The culture supernatant containing the non- adherent mononuclear cells was removed, and the adherent cells were gently washed with DMEM. The DMEM was discarded and fresh PM was added to each flask containing the plastic adherent MSC cells. The process phase from MNC seeding to MSC harvesting was designated Passage 0 (P0).

The P0 cells were incubated in a 37°C/5% CO_2 humidified incubator and PM was replaced twice a week, with fresh PM, until the culture was sub-confluent.

Propagation of MSC: Primary cultures of MSC were grown *in-vitro* as a single cell layer attached to a plastic substrate. Once the available substrate surface was covered by cells (a confluent culture), growth slowed and then ceased. Thus, in order to keep the cells healthy and actively growing, it was necessary to subculture them at regular intervals, when the culture was sub-confluent. Each subculture cycle is designated Passage. The MSC culture was passaged at a density of 500-2,000 cells/cm^2.

For passaging MSC, the culture supernatant was removed from the flask and Trypsin (TrypLE™ Select, Invitrogen) was added to each flask. The flask was incubated for several minutes at 37 ºC and the resulting cell suspension was collected from the flask into centrifuge tubes and DMEM was added to each flask for diluting the Trypsin and collecting the remaining cells.

The cell suspension was centrifuged re-suspended in PM, counted and reseeded at a density of 500-2,000 cells/cm^2 in new culture vessels. The cultures were then incubated in a 37ºC/5% CO_2 humidified incubator.

In the course of each passage the PM was replaced every 3-4 days, by removing all the culture supernatant and replacing it with the same volume of fresh PM.

Induction of differentiation: MSC were seeded for induction of differentiation in PM at a concentration of about 6,000-8,000 cells/cm^2. Three days later, differentiation was induced by replacing the PM with differentiation medium (S2M) containing low glucose DMEM supplemented with 1mM dibutyryl cyclic AMP (cAMP), 20 ng/ml human Basic Fibroblast Growth Factor (hbFGF), 5 ng/ml human platelet derived growth factor (PDGF-AA), and 50 ng/ml human Heregulin β1. The culture was maintained in differentiation medium for 3 days until harvesting.

MSC-NTF cells were harvested 24 hours before the end of differentiation (Day 2) and/or at the end of differentiation (Day 3). MSC cell were harvested from the same donor or patient at the same passage at the same time.

Sample Preparation, acquisition and analysis: Cells were suspended in PBS at a concentration of 0.5-1x10^6 cells/tube and stained for 30 minutes on ice with a mouse monoclonal Antibody to Integrin α1 (IgG1, clone TS2/7.1.1, Santa Cruz Cat# SC-81733PE). The isotype control was a Mouse IgG1 k - PE conjugated, isotype control (clone MOPC-21, Cat# 555749 BD Biosciences). Cells were analyzed by Flow

Cytometry (Cytomics FC 500, Beckman Coulter, Inc.) and the data analyzed using the CXP software (Beckman Coulter, Inc.).

RESULTS

The expression of Integrin alpha 1 (CD49a) was studied on the surface of MSC and MSC cells induced to secrete neurotropic factors (MSC-NTF cells).

At the end of the differentiation process (Day 3) about 90 ± 4.43 % (mean ± standard deviation) of the MSC-NTF cell population expressed CD49a as compared to 68.75± 4.29 % (mean ± standard deviation) of the non-differentiated MSC cell population of the same donor (n=8). The difference between the two populations is highly significant ($p<0.0001$, Day 3, Table 1A).

On Day 3, Mean Fluorescence Intensity (MFI) was also found to significantly increase in MSC-NTF cells populations from 2.75 ± 0.48 % (mean ± standard deviation) of MSC to 13.2 ± 4.77% (mean ± standard deviation) for MSC-NTF cells, an average 4.87±1.56 fold induction (n=8, Table 1A).

One day prior to the end of differentiation (Day 2) 90.55 ± 6.62 % (mean ± standard deviation) of the MSC-NTF cell populations expressed CD49a as compared to 73 ± 6 % (mean ± standard deviation) of the non-differentiated MSC cell population of the same donor. The difference between the two populations is highly significant ($p<0.005$, Day 2, Table 1A).

On Day 2, Mean Fluorescence Intensity (MFI) was also found to significantly increase in MSC-NTF cells populations from 2.84 ± 0.98 % (mean ± standard deviation) of MSC to 11.58 ± 7.18 % (mean ± standard deviation) for MSC-NTF cells an average 3.77±1.43 fold induction (n=4, Table 1A).

Table 1A

	MSC		MSC-NTF		p value for difference in	MFI Fold	
	% Positives	MFI	% Positives	MFI	% positives	induction	n
Day 2	73 ± 6	2.84 ± 0.98	90.55 ± 6.62	11.58 ± 7.18	p<0.005	3.77±1.43	4
Day 3	68.75±4.29	2.75 ± 0.48	90 ± 4.43	13.2 ± 4.77	p<0.0001	4.87±1.56	28

Two additional experiments were performed to corroborate these results. For the first experiment, at the end of the differentiation process (Day 3), about 80.9 % of the MSC-NTF cell population expressed CD49a as compared 56.05 % of the non-differentiated MSC cell population of the same donor (Table 1B). For the second experiment, at the end of the differentiation process (Day 3) about 89 % of the MSC-

NTF cell population expressed CD49a as compared 60 % of the non-differentiated MSC cell population of the same donor (Table 1B).

Table 1B

	CD49a % positives	
Exp#	MSC	MSC-NTF
1	56.05	80.9
2	60	89

EXAMPLE 2

Comparison of MSC-NTFs with non-differentiated MSCs

MATERIALS AND METHODS

Induction of Differentiation: as detailed in Example 1.

Measurement of Growth/differentiation factor-15 (GDF-15) and Interleukin 8 (IL-8) The amount of GDF-15 and IL-8 in the cell's culture supernatants at the end of differentiation were quantified by using the GDF-15 ELISA assay (GDF-15 DuoSet DY957; R&D Systems) the IL-8 ELISA assay (IL-8 DuoSet DY208; R&D Systems) according to the manufacturer's protocol, for example and without limitation.

Proteomics

Proteolysis: The protein were extracted from the cell pellets in 9M Urea, 400 mM Ammonium bicarbonate and 10 mM DTT and two cycles of sonication. 20 µg protein from each sample were reduced with 2.8 mM DTT (60°C for 30 min), modified with 8.8 mM iodoacetamide in 400 mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 2 M Urea, 25 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37 °C. An additional second trypsinization was done for 4 hours.

Mass spectrometry analysis: The tryptic peptides were desalted using C18 tips (Harvard) dried and re-suspended in 0.1% Formic acid.

The peptides were resolved by reverse-phase chromatography on 0.075 X 180-mm fused silica capillaries (J&W) packed with Reprosil® reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 180 minutes gradient of 5 to 28 % 5 minutes gradient of 28 to 95 % and 25 minutes at 95 % acetonitrile with 0.1% formic acid in water at flow rates of 0.15 µl/min. Mass spectrometry was performed by Q Exactive plus mass spectrometer (Thermo) in a positive mode using

repetitively full MS scan followed by collision induces dissociation (CID) of the 10 most dominant ions selected from the first MS scan.

The mass spectrometry data from three biological repeats was analyzed using the MaxQuant software 1.4.1.2 (Mathias Mann's group) vs. the human section of the Uniprot database with 1% FDR. The data was quantified by label free analysis using the same software.

The intensity data was transformed to log 2 in order to get a normal distribution.

Welch T-Test with Permutation-based FDR, (with 250 randomization, Threshold value=0.05) between the A and the B groups was done using the Preseuse 1.4. Same software was used for additional annotations and data correlation.

Genearray: Genearray analyses were run using the Expression Array Gene ST 2.0 GeneChip® Human Gene 2.0 ST Array (Affymetrix).

Cell pellets were resuspended in RNA Protect (Qiagen). Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen, cat#74134). RNA Quality was measured using TapeStation (Agilent). 250ng of RNA were labeled using GeneChip® WT PLUS Reagent Kit (Affymetrix, cat# 902280), following manufacturer manual (Affymetrix cat# 703174 Rev. 2). Briefly, cDNA was synthesized from the RNA using random primers, while adding a T7 promoter tail. cRNA was then generated by in vitro transcription using T7-RNA-Polymerase. Single-stranded cDNA was synthesized, then fragmented and end-labeled. 3.5ug were hybridized to a GeneChip® Human Gene 2.0 ST Arrays (Affymetrix, cat#902499). Arrays were washed and stained using the GeneChip Hybridization Wash and Stain kit (Affymetrix cat#900720) and scanned. Images were subjected to visual inspection, followed by quantitation (RMA-gene), normalization (Sketch-Quantile) and QC using Expression Console build 1.3.1.187 (Affymetrix). All parameters passed QC metrics and no outliers were observed. A list of deferentially expressed genes was generated using One-Way Between-Subject ANOVA (Unpaired) with the Transcriptome Analysis Console 2.0 (Affymetrix).

The experiment compared the untreated MSC control and MSC-NTF cells induced to differentiate by the one step protocol described for Example 1. Samples from three unrelated subjects were analyzed for each condition. The overall difference between individuals was found to be smaller than between conditions.

RESULTS

It was found that in 23 ALS patients, specific productivity of GDF-15 was in the range of 225.79 ± 99.72 pg/ml/x10^6 cells in MSC and was found to increase to 1257.20 ± 890.60 pg/ml/x10^6 in MSC-NTF cells of the same patient prior to differentiation, a 9.4 fold average increase (Figure 2A).

Further, it was found that MSC-NTF cells of ALS patients secreted significant amounts of IL-8 (an average of 81 ± 43 ng/ml/x10^6 cells) as compared to MSC of the same patient prior to differentiation, a 170 fold average increase (Figure 2B).

Bone marrow derived MSCs from ALS patients were analyzed via proteomics both prior to and following differentiation using the protocol described in the materials and methods.

The most significantly up- or down-regulated proteins, based on identification by at least two peptides in three repeats using Mass spec, normalized for the intensity of the detection of the protein are presented in Table 2, herein below.

Table 2

	Protein IDs	***Protein names***	***Gene names***	***welch p value_PA1 vs PB1***	***welch Difference_PA1 vs PB1***
1	Q04828;H0Y804; A6NHU4;P17516	Aldo-keto reductase family 1 member C1	AKR1C1	0.025239	-4.27165
2	P36222;H0Y3U8	Chitinase-3-like protein 1	CHI3L1	0.00333	-8.03492
3	O14684	Prostaglandin E synthase	PTGES	0.03174	-6.82836
4	P09601;B1AHA8	Heme oxygenase 1	HMOX1	0.004332	-3.58814
5	Q16678	Cytochrome P450 1B1	CYP1B1	0.003662	-7.19957
6	Q7LBR1	Charged multivesicular body protein 1b	CHMP1B	0.003375	-4.41248
7	Q9BS40	Latexin	LXN	0.01834	-3.01403
8	P01033;Q5H9A7; H0Y789;Q5H9B5; Q5H9B4	Metalloproteinase inhibitor 1	TIMP1	0.007288	-3.62584

9	P48307;H7C4A3	Tissue factor pathway inhibitor 2	TFPI2	1	ND
10	Q8WUJ3;H0YL56;H0YCE1	Protein KIAA1199	KIAA1199	0.014234	-3.67028
11	P42330;S4R3Z2;S4R3D5	Aldo-keto reductase family 1 member C3	AKR1C3	0.02981	-3.86375
12	P17301	Integrin alpha-2	ITGA2	0.001484	-5.15083
13	O94875;H7BZK1;Q9BX66	Sorbin and SH3 domain-containing protein 2	SORBS2	0.013196	-4.11166
14	P52895;B4DK69;S4R3P0	Aldo-keto reductase family 1 member C2	AKR1C2	0.026833	-4.96764
15	P41221;C9J8I8;Q9H1J7;F5H7Q6;F5H364;F5H034;O00755	Protein Wnt-5a	WNT5A	0.010376	-3.1782
16	Q9HCJ1;D6RGI5	Progressive ankylosis protein homolog	ANKH	0.018078	-3.77482
17	P07093;C9JN98;C9K031	Glia-derived nexin	SERPINE2	0.005989	-3.61762
18	Q5VYS4;H0Y831	Mesenteric estrogen-dependent adipogenesis protein	MEDAG	0.003767	-4.08412
19	Q13228;H0Y532;A6PVX1;F2Z2W8;F8WCR4;C9JVL0;F8WBA9	Selenium-binding protein 1	SELENBP1	0.024781	-3.34893
20	O00194;K7ES41;K7EJ38	Ras-related protein Rab-27B	RAB27B	0.023078	-3.70819
21	P17676	CCAAT/enhancer-binding protein beta	CEBPB	0.008846	-3.43367
22	Q99988	Growth/differentiation factor 15	GDF15	1	ND
23	Q8IWB1;X6RK76	Inositol 1,4,5-trisphosphate receptor-interacting protein	ITPRIP	0.003773	-5.21981
24	P02675;D6REL8;CON__P02676	Fibrinogen beta chain;Fibrinopeptide B;Fibrinogen beta chain	FGB	1	-6.5007
25	P10253;I3L0S5;I3L3L3	Lysosomal alpha-glucosidase;76 kDa	GAA	0.011612	-3.59936

		lysosomal alpha-glucosidase;70 kDa lysosomal alpha-glucosidase			
26	C9JEU5;P02679;C9JC84;C9JPQ9;C9JU00	Fibrinogen gamma chain	FGG	0.012628	-6.08619
27	Q8IV20;A2A3Z5	Laccase domain-containing protein 1	LACC1	0.044734	-4.52527
28	Q96AQ6;Q5T173	Pre-B-cell leukemia transcription factor-interacting protein 1	PBXIP1	0.004564	-3.39635
29	O15118;K7EQ23	Niemann-Pick C1 protein	NPC1	0.010158	-4.33114
30	Q7Z2X4	PTB-containing, cubilin and LRP1-interacting protein	PID1	0.026791	-4.2971
31	P61587;E9PFH1;Q53RZ3	Rho-related GTP-binding protein RhoE	RND3	0.029183	-3.05365
32	P32189;Q14409;F8WC39;A6NP46;C9JLT1;F8WDA9;F8WBI8;F8WF44;H7BYD2;H7C2A0	Glycerol kinase; Putative glycerol kinase 3	GK;GK3P	0.018576	-4.98535
33	P04003;A6PVY5;F2Z2V7	C4b-binding protein alpha chain	C4BPA	1	-5.46453
34	Q8N726	Cyclin-dependent kinase inhibitor 2A, isoform 4	CDKN2A	1	ND
35	A0JP02;B4DJX4;Q9HAU0;E7EME8;H0YG48;H0YGJ6	Pleckstrin homology domain-containing family A member 5	PLEKHA5	0.022674	-3.89935
36	P43003;M0R063;P48664;E7EUS7;E7EUV6;M0QY32;M0R106;E7EV13	Excitatory amino acid transporter 1	SLC1A3	0.036812	-3.63458
37	F5GYK4;O00142;E5KNQ5;H3BP77;J3KS73;J3QL12;J3QRP0;J3KRW8	Thymidine kinase 2, mitochondrial	TK2	0.00388	-4.92515
38	P98066	Tumor necrosis factor-inducible gene 6 protein	TNFAIP6	1	ND
39	P06280;V9GYN5	Alpha-galactosidase A	GLA	0.047612	-4.14468
40	P38936;J3KQ	Cyclin-dependent	CDKN1	1	ND

	V0	kinase inhibitor 1	A		
41	P08476	Inhibin beta A chain	INHBA	1	ND
42	Q9UHE8	Metalloreductase STEAP1	STEAP 1	1	ND
43	Q8WWI5	Choline transporter-like protein 1	SLC44 A1	0.009593	-3.14327
44	P01024;CON_ _Q2UVX4;M0 R0Q9;M0QYC 8;M0QXZ3	Complement C3; Complement C3 beta chain;Complement C3 alpha chain;C3a anaphylatoxin; Acylation stimulating protein;Complement C3b alpha chain; Complement C3c alpha chain fragment 1; Complement C3dg fragment;Complement C3g fragment; Complement C3d fragment;Complement C3f fragment; Complement C3c alpha chain fragment 2	C3	0.01925	-4.77651
45	Q9ULG6;H3B N32	Cell cycle progression protein 1	CCPG1	0.028133	-3.63365
46	P05165;H0Y5 U0;Q5JTW6	Propionyl-CoA carboxylase alpha chain, mitochondrial	PCCA	0.023694	-3.97258
47	P14923	Junction plakoglobin	JUP	0.001268	-4.30589
48	Q9Y4F1;C9JM E2;M0QXT1; M0QYB0;H0Y 783;M0R262	FERM, RhoGEF and pleckstrin domain-containing protein 1	FARP1	0.014285	-3.70348
49	Q9NRZ5;Q6A I25;G3XAF1; Q5TEE8	1-acyl-sn-glycerol-3-phosphate acyltransferase delta	AGPAT 4	0.00875	-3.07989
50	Q9H098;C9J6 N5;C9JQ40;C9 JW51;C9J3Q3; X6RET8;C9JP 05;F8WCJ2;C 9JYP1;C9J6Y8	Protein FAM107B	FAM10 7B	1	ND
51	P18428	Lipopolysaccharide -binding protein	LBP	0.021616	-3.41834
52	H0Y4R5;Q5S NT2	Transmembrane protein 201	TMEM 201	1	ND
53	Q8N6G5	Chondroitin sulfate N-acetylgalactosaminyl transferase 2	CSGAL NACT2	1	ND
54	Q8NFT2;B5M	Metalloreductase	STEAP	0.033114	-3.16521

	C02;C9JLP2	STEAP2	2		
55	P35475;D6REB5;H0Y9B3;D6R9D5;D6RBD5;H0Y9R9	Alpha-L-iduronidase	IDUA	1	ND
56	P05546	Heparin cofactor 2	SERPIND1	1	-3.13676
57	H7BXR3;H7C1R7;H7BZX1;C9J3W4;C9JL62;C9IZ89		SORBS2	1	ND
58	Q5QJ74;E9PP54;E9PNS0;E9PJJ0;B3KNB6;G3V147	Tubulin-specific chaperone cofactor E-like protein	TBCEL	1	ND
59	P36269;H7C1X2	Gamma-glutamyltransferase 5;Gamma-glutamyltransferase 5 heavy chain;Gamma-glutamyltransferase 5 light chain	GGT5	1	ND
60	Q13219	Pappalysin-1	PAPPA	1	ND
61	Q9P2B2	Prostaglandin F2 receptor negative regulator	PTGFRN	1	ND
62	E7EW77;E7EP65;Q9NYB9;J3KNB2;E9PEZ7;H7C3Q7;H0Y6B5;F8WBL5;F8WAQ3;F8WEB9;E7EUA1;F8WAU3;F8WCD7;F8WAZ8	Abl interactor 2	ABI2	1	ND
63	P58335	Anthrax toxin receptor 2	ANTXR2	0.017357	-3.75924
64	P51884;CON__Q05443	Lumican	LUM	1	ND
65	Q86UX7;F5H1C6;F5H3I6	Fermitin family homolog 3	FERMT3	1	ND
66	P35869;E5RGQ2;G3V143;E5RFG4;A9YTQ3	Aryl hydrocarbon receptor	AHR	0.029594	-3.42358
67	P56199	Integrin alpha-1	ITGA1	1	ND
68	P35354;Q6ZYK7	Prostaglandin G/H synthase 2	PTGS2	1	ND
69	Q96MK3	Protein FAM20A	FAM20A	1	ND
70	Q96CC6;F8WCF7;B8ZZ07;F	Inactive rhomboid protein 1	RHBDF1	1	ND

	6XBT0;F8WBS4				
71	P33897;H0Y7L9	ATP-binding cassette sub-family D member 1	ABCD1	1	-3.15122
72	Q05707;J3QT83;Q4G0W3	Collagen alpha-1(XIV) chain	COL14A1	0.047499	-3.24857
73	P43490;Q5SYT8;F5H246;C9JG65;C9JF35	Nicotinamide phosphoribosyltransferase	NAMPT;NAMPTL	0.010112	-2.84468
74	P17302	Gap junction alpha-1 protein	GJA1	0.005458	-2.72582
75	P07711;Q5T8F0;Q5NE16;O60911	Cathepsin L1;Cathepsin L1 heavy chain;Cathepsin L1 light chain	CTSL1	0.023268	-2.64752
76	P11498;E9PRE7;E9PS68	Pyruvate carboxylase, mitochondrial	PC	0.002932	-2.77116
77	P17677	Neuromodulin	GAP43	0.041471	-2.55059
78	Q7LG56;H0YAV1	Ribonucleoside-diphosphate reductase subunit M2 B	RRM2B	0.015477	-2.57625
79	Q02252;G3V4Z4	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	ALDH6A1	0.00472	-2.98955
80	E9PF16;Q96CM8;D6RF87	Acyl-CoA synthetase family member 2, mitochondrial	ACSF2	0.036886	-2.88375
81	P23786	Carnitine O-palmitoyltransferase 2, mitochondrial	CPT2	0.040208	-2.55565
82	C9JGI3;P19971	Thymidine phosphorylase	TYMP	0.032844	-2.93769
83	F5H6B2;Q9UHN6	Transmembrane protein 2	TMEM2	1	ND
84	P10915;D6RBS1	Hyaluronan and proteoglycan link protein 1	HAPLN1	1	ND
85	Q6UVK1	Chondroitin sulfate proteoglycan 4	CSPG4	1	ND
86	P01130;J3KMZ9;H0YMD1;H0YMQ3;H0YM92	Low-density lipoprotein receptor	LDLR	1	ND
87	G3V511;G3V3X5;Q14767	Latent-transforming growth factor beta-binding protein 2	LTBP2	1	4.404051
88	F5H855;P56945;Q14511	Breast cancer anti-estrogen resistance protein 1	BCAR1	1	4.176916

89	O95801;Q5TA95	Tetratricopeptide repeat protein 4	TTC4	1	ND
90	O95347;Q5T821	Structural maintenance of chromosomes protein 2	SMC2	1	ND
91	P43007	Neutral amino acid transporter A	SLC1A4	1	ND
92	P26022	Pentraxin-related protein PTX3	PTX3	1	3.989578
93	Q8TB03	Uncharacterized protein CXorf38	CXorf38	1	ND
94	Q8IZ07;S4R3D2;H0YIN8;F8W150;S4R3U2;Q6ZTN6	Ankyrin repeat domain-containing protein 13A	ANKRD13A	1	5.833572
95	Q9NX58	Cell growth-regulating nucleolar protein	LYAR	1	ND
96	P02790	Hemopexin	HPX	1	ND
97	Q6ZN40;H0YL80;H0YLS7		TPM1	0.004913	3.131315
98	P08123	Collagen alpha-2(I) chain	COL1A2	0.008003	3.385308
99	K7ENT6;K7ERG3		TPM4	0.034608	3.634858
100	P02452;CON__Q862S4;I3L3H7;P02458	Collagen alpha-1(I) chain	COL1A1	0.008651	5.24923
101	P20337	Ras-related protein Rab-3B	RAB3B	0.00338	3.365877
102	P0C0L5;F5GXS0	Complement C4-B;Complement C4 beta chain;Complement C4-B alpha chain;C4a anaphylatoxin;C4b-B;C4d-B;Complement C4 gamma chain	C4B	0.035673	4.70512
103	Q14566	DNA replication licensing factor MCM6	MCM6	0.015309	3.802778
104	Q9H7C4;C9JTN4;C9JSS1	Syncoilin	SYNC	0.04319	3.910519
105	P02787;C9JVG0;H7C5E8;F8WEK9;F8WCI6;C9JB55;F8WC57;CON__Q29443;CON__Q0IIK2	Serotransferrin	TF	0.013404	6.25542
106	P49736;H7C4N9;C9J013;C9JZ21;F8WDM3	DNA replication licensing factor MCM2	MCM2	0.040152	4.184367
107	P33993;C9J8	DNA replication	MCM7	0.035577	3.315128

	M6	licensing factor MCM7			
108	P33991;E5RG31;E5RFJ8;E5RFR3	DNA replication licensing factor MCM4	MCM4	0.033108	3.523307
109	P25205;B4DWW4;J3KQ69;Q7Z6P5	DNA replication licensing factor MCM3	MCM3	0.040364	3.55418
110	E9PD53;Q9NTJ3;C9JR83;C9JVD8;C9J578;C9J9E4	Structural maintenance of chromosomes protein;Structural maintenance of chromosomes protein 4	SMC4	0.011999	3.016034
111	P26006;H0YA49;H0YA32;K7EMU3;D6R9X8	Integrin alpha-3;Integrin alpha-3 heavy chain;Integrin alpha-3 light chain	ITGA3	0.030181	3.334243
112	P01023;CON__ENSEMBL:ENSBTAP00000024146;P20742;H0YFH1;F8W7L3;F5H1E8	Alpha-2-macroglobulin	A2M	0.000365	8.539567
113	O95361;B3KP96;H0Y626;K7ENN8;Q309B1;K7EL43;I3L3K9;I3L2F3;J3QL38;J3QKY5	Tripartite motif-containing protein 16	TRIM16	1	3.605912
114	P05121	Plasminogen activator inhibitor 1	SERPINE1	1	5.247232
115	Q15021;E7EN77	Condensin complex subunit 1	NCAPD2	0.041189	4.251724
116	H7BYY1;F5H7S3;B7Z596;H0YL42;H0YK20		TPM1	0.008933	2.809683
117	P08243;F8WEJ5;C9J057;C9JT45;C9JM09;C9JLN6	Asparagine synthetase [glutamine-hydrolyzing]	ASNS	0.011498	2.974403
118	O43294;H3BQC4;H3BSN4;I3L209;H3BS04;H3BN49	Transforming growth factor beta-1-induced transcript 1 protein	TGFB1I1	0.014001	2.818781
119	P20908;H7BY82;P12107;C9JMN2;H0YIS1;Q4VXY6;P13942;P25940	Collagen alpha-1(V) chain	COL5A1	0.019362	2.800896
120	Q5H909;Q9UNF1;Q5H907	Melanoma-associated antigen D2	MAGED2	0.011154	2.607765
121	P35520;C9JMA6;H7C2H4	Cystathionine beta-synthase;Cysteine	CBS	0.029219	2.717574

		synthase			
122	P23921;E9PL69	Ribonucleoside-diphosphate reductase large subunit	RRM1	0.033696	2.537958

Bone marrow derived MSCs from ALS patients were analyzed via Genearray both prior to and following differentiation using the protocol described in the materials and methods.

Out of a total of 48,226 genes that were analyzed, 1623 genes were found to be differentially expressed - 518 genes were found to be up-regulated and 567 genes were found to be down-regulated.

Table 3, herein below provides a list of exemplary genes that were significantly up or down regulated following differentiation.

Table 3

	Fold Change (linear)	ANOVA p-value	Gene Symbol	Description
1	87.97	0.000045	SLC16A6	solute carrier family 16, member 6 (monocarboxylic acid transporter 7); NULL
2	66.16	0.000142	IL8	interleukin 8
3	48.62	0.000019	MMP13	matrix metallopeptidase 13 (collagenase 3)
4	47.05	0.000048	BMP2	bone morphogenetic protein 2
5	37.07	0.002339	CXCL6	chemokine (C-X-C motif) ligand 6
6	30.74	0.000005	RASD1	RAS, dexamethasone-induced 1
7	29.67	0.000006	IL11	interleukin 11
8	28.33	4.29E-07	PCSK1	proprotein convertase subtilisin/kexin type 1
9	27.52	0.001772	TFPI2	tissue factor pathway inhibitor 2
10	27.43	0.001103	AREG	amphiregulin; amphiregulin B
11	26.99	0.000544	PTGES	prostaglandin E synthase
12	26.35	0.004312	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)
13	25.85	0.009822	CXCL5	chemokine (C-X-C motif) ligand 5
14	22.3	0.000954	AREGB	amphiregulin B; amphiregulin
15	22.18	0.000314		
16	21.76	0.000641	COL10A1	collagen, type X, alpha 1
17	20.32	0.007707		
18	16.09	0.000037	PTHLH	parathyroid hormone-like hormone
19	15.1	0.000566	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
20	14.88	0.00001	SMOC1	SPARC related modular calcium binding 1
21	13.65	0.000138	ABCA1	ATP-binding cassette, sub-family A

				(ABC1), member 1
22	13.59	0.000006	MEDAG	mesenteric estrogen-dependent adipogenesis
23	12.67	0.000898	OTTHUMG00000037425	NULL
24	12.49	0.000627	ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6; NULL
25	12.25	0.000573	IL1B	interleukin 1, beta; NULL
26	11.53	0.007657	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase); NULL
27	11.52	0.000001	SMOX	spermine oxidase; NULL
28	11.46	0.000021	GAS1	growth arrest-specific 1
29	11.45	0.000237		
30	11.39	0.000207	CXCL16	chemokine (C-X-C motif) ligand 16; NULL
31	10.87	0.00025	PITPNC1	phosphatidylinositol transfer protein, cytoplasmic 1
32	10.6	0.000128	NR4A2	nuclear receptor subfamily 4, group A, member 2
33	10.56	0.000062	FZD8	frizzled family receptor 8; microRNA 4683
34	10.11	0.037241	MIR3189	microRNA 3189
35	10.03	0.000307	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5
36	9.86	0.002102	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
37	9.62	0.000104	LIF	leukemia inhibitory factor
38	9.42	0.000944	RAB27B	RAB27B, member RAS oncogene family
39	9.39	0.000091	GEM	GTP binding protein overexpressed in skeletal muscle
40	9.06	0.000311		
41	9.03	0.000797	HAS1	hyaluronan synthase 1
42	-8.96	0.006205	CTGF	connective tissue growth factor
43	-9.3	0.024107	KRTAP2-3	keratin associated protein 2-3; keratin associated protein 2-4
44	-9.78	0.000051	TOP2A	topoisomerase (DNA) II alpha 170kDa
45	-10.56	0.000014	PBK	PDZ binding kinase
46	-10.63	0.000022	TPX2	TPX2, microtubule-associated, homolog (Xenopus laevis)
47	-11.27	0.003641		
48	-11.48	0.000012	DLGAP5	discs, large (Drosophila) homolog-associated protein 5
49	-12.52	0.000018	CCNA2	cyclin A2
50	-12.72	0.002789		
51	-14.5	0.000007	ANLN	anillin, actin binding protein; NULL
52	-14.75	0.002755	CYR61	cysteine-rich, angiogenic inducer, 61

53	-15.07	0.000217	B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2
54	-15.21	0.001511	ALPL	alkaline phosphatase, liver/bone/kidney; NULL
55	-18.93	0.000415	TAGLN	Transgelin
56	-27.82	0.005062	PTX3	pentraxin 3, long

EXAMPLE 3

Comparison of MSC-NTFs using two different differentiation protocols

MATERIALS AND METHODS

Differentiation protocol 1: As described in Example 1 - this is referred to herein as the one-step protocol.

Differentiation protocol 2: As described in WO2009/144718 - this is referred to herein as the two-step protocol.

In short, human MSC (12,000 cells/cm^2) were first placed in DMEM supplemented with SPN, 2mM L-Glutamine (Biological industries), 20 ng/ml human epidermal growth factor (hEGF), 20 ng/ml human basic fibroblast growth factor (hbFGF) (R&D Systems) and N2 supplement (Invitrogen). After 72 hours, the medium was replaced with DMEM supplemented with 1mM dibutyryl cyclic AMP (dbcAMP), 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 5 ng/ml human platelet derived growth factor (PDGF), 50 ng/ml human neuregulin 1-β1/ HRG1-β1 EGF domain and 20 ng/ml hbFGF (all from R&D Systems) for 3 more days.

Proteomics: Performed as described in Example 2.

Gene array analyses: Performed as described in Example 2.

The experiment compared MSC-NTF cells induced to differentiate by the one step protocol or by the two step protocol. Samples from three unrelated subjects were analyzed for each condition. The overall difference between individuals was found to be smaller than between conditions. The overall difference between each differentiation protocol and the control was found to be greater than between the protocols.

RESULTS

The most significantly up- or down-regulated proteins identified when comparing MSC cells differentiated by the two protocols, based on identification by at least two peptides in three repeats using Mass spec, normalized for the intensity of the detection of the protein are presented in Table 4, herein below.

Table 4

	Protein ID	*Majority Protein ID*	*Protein Name*	*Gene name*	*Welch P Value*	*Welch difference*
1	Q9BVA1	Q9BVA1	Tubulin beta-2B chain	TUBB2B	0.000583	3.441701
2	Q15392; H7C4B7	Q15392	Delta(24)-sterol reductase	DHCR24	0.013112	-3.09319
3	S4R371;P05413;S4R3A2	S4R371;P05413;S4R3A2	Fatty acid-binding protein, heart	FABP3	0.03411	-3.10571
4	E7EVP7;Q14643;B7ZMI3	E7EVP7;Q14643	Inositol 1,4,5-trisphosphate receptor type 1	ITPR1	0.00456	-3.58643
5	O95757;E9PDE8;D6RJ96	O95757;E9PDE8;D6RJ96	Heat shock 70 kDa protein 4L	HSPA4L	0.040802	-3.66797
6	G5E9F5;B5MC53;B5MCF8;P39210;C9J473;H0Y6M5;E7EX18	G5E9F5;B5MC53;B5MCF8;P39210;C9J473;H0Y6M5;E7EX18	Protein Mpv17	MPV17	0.002826	-6.1913
7	Q8N2G8;K7ESN3	Q8N2G8;K7ESN3	GH3 domain-containing protein	GHDC	0.000996	-3.30843
8	P08236;F8WBK6;F2Z3L6	P08236	Beta-glucuronidase	GUSB	0.014338	-3.70965
9	H3BUL4;H3BMX9;Q9GZU8;H3BQQ6;H3BTI2;H3BTP8;H3BSY6;H3BP64;H3BU93;Q6P4H7;H3BSF0;H3BNK9	H3BUL4;H3BMX9;Q9GZU8;H3BQQ6;H3BTI2;H3BTP8;H3BSY6;H3BP64;H3BU93	Protein FAM192A	FAM192A	0.022477	3.316088

Out of a total of 48,226 genes that were analyzed, 100 genes were found to be up-regulated in the two step protocol as compared to the one step protocol and 69 genes were found to be down-regulated in the two step protocol as compared to the one step protocol.

Table 5, herein below provides a list of exemplary genes that were significantly up or down regulated following differentiation.

Table 5

	Transcript Cluster ID	Protocol2 Bi-weight Avg Signal (log2)	Protocol1 Bi-weight Avg Signal (log2)	Fold Change (linear) (Protocol2 vs. Protocol1)	ANOVA p-value (Protocol2 vs. Protocol1)	FDR p-value (Protocol2 vs. Protocol1)	Gene Symbol
1	16913537	9.31	6.4	7.54	0.002082	0.394067	LBP
2	17080	11.59	8.91	6.44	0.000435	0.26432	ENPP2

	516						
3	16908197	11.76	9.29	5.51	0.00794	0.474763	IGFBP5
4	16919242	10.33	7.91	5.34	0.004	0.439362	MAFB
5	17075789	8	5.74	4.77	0.000235	0.227658	SCARA5
6	16716371	10.36	8.2	4.49	0.004162	0.440384	CH25H
7	16691327	7.75	5.67	4.23	0.004869	0.444802	NGF
8	16816034	11.72	9.65	4.21	0.00035	0.263671	NPIPA1
9	16795943	6.39	4.34	4.13	0.005322	0.450478	TC2N
10	16944010	9.12	7.14	3.93	0.00011	0.198375	BOC
11	16782003	6.6	4.62	3.93	0.01791	0.583076	TRDV3
12	16863593	6.96	5.04	3.8	0.000775	0.319307	C5AR2
13	16816287	11.37	9.47	3.73	0.000126	0.198375	PKD1P1
14	16824400	11.84	9.95	3.7	0.000078	0.198375	NPIPA5
15	16676988	8.61	6.73	3.68	0.018038	0.583776	HSD11B1
16	16853879	6.92	5.09	3.56	0.017063	0.575446	PIEZO2
17	16774384	10.24	8.44	3.49	0.001238	0.353527	TNFSF11
18	16888669	4.22	2.42	3.46	0.009271	0.497877	MIR1245A
19	16980762	8.43	6.68	3.37	0.001297	0.353527	SFRP2
20	16730967	8.12	6.37	3.37	0.001278	0.353527	C11orf87
21	16814986	8.63	6.91	3.29	0.049553	0.722228	MIR4516
22	16824127	9.21	7.53	3.2	0.004523	0.440937	LOC100288162
23	16729290	11.15	9.5	3.15	0.000002	0.02648	TSKU
24	16936947	8.33	6.69	3.12	0.029614	0.660051	ITPR1
25	16840113	10.27	8.65	3.07	0.030963	0.664666	CXCL16
26	16742384	7.95	6.34	3.04	0.00703	0.472433	LRRC32
2	16824	11.58	9.97	3.03	0.000113	0.198375	PKD1P

7	366						1
28	16996234	9.47	7.89	3	0.003441	0.414166	PPAP2A
29	16824193	12.35	10.81	2.9	0.000085	0.198375	NPIPA5
30	16774427	11.1	9.59	2.83	0.03554	0.675853	LACC1
31	16774303	5.41	3.92	2.81	0.013365	0.537579	RGCC
32	16994002	8.38	6.92	2.75	0.000489	0.275839	LPCAT1
33	16756447	6.35	4.91	2.71	0.004243	0.440384	BTBD11
34	17043843	8.43	6.99	2.71	0.032515	0.666018	TSPAN13
35	17087109	6.24	4.82	2.68	0.009133	0.497794	MIR27B
36	16970435	8.54	7.14	2.65	0.003122	0.410198	SPRY1
37	16816343	12.62	11.21	2.65	0.000222	0.227658	NPIPA1
38	16910070	7.35	5.95	2.64	0.016769	0.571533	MIR4441
39	16723020	6.53	5.14	2.62	0.003207	0.413056	ANO3
40	16825484	10.52	9.2	2.5	0.003972	0.438334	NPIPB3
41	16746930	9.68	8.36	2.49	0.005319	0.450478	TSPAN9
42	16893349	10.77	9.46	2.48	0.009614	0.504379	SNED1
43	16824166	9.15	7.86	2.44	0.00181	0.383976	LOC399491
44	16871235	7.62	6.34	2.43	7.56E-07	0.02648	CEBPA
45	17088462	11.37	10.11	2.41	0.005785	0.456611	PAPPA
46	16890675	7.61	6.34	2.41	0.012505	0.529023	IGFBP2
47	16920047	9.28	8.03	2.39	0.003573	0.414166	PREX1
48	17005077	8.19	6.95	2.36	0.01056	0.508075	MYLIP
49	16824349	8.83	7.59	2.36	0.000113	0.198375	LOC100288162
50	16661646	10.07	8.84	2.35	0.005955	0.457174	RNU11
51	16976827	11.73	10.5	2.34	0.036147	0.67781	CXCL5
5	16970	7.83	6.61	2.34	0.004538	0.440937	FAT4

2	465						
53	16661730	10.28	9.06	2.33	0.016407	0.567596	PTPRU
54	16990203	6.9	5.69	2.32	0.04901	0.722228	VTRNA1-3
55	16795965	8.98	7.77	2.31	0.003339	0.414166	FBLN5
56	17012140	5.05	3.86	2.29	0.048905	0.722228	RNA5SP215
57	16886174	7.46	6.27	2.28	0.017839	0.582777	KYNU
58	16802497	7.05	5.86	2.28	0.005396	0.450478	PAQR5
59	17023799	9.17	8.01	2.24	0.025425	0.640796	SLC2A12
60	16998059	10.49	9.34	2.22	0.009737	0.506539	ARRDC3
61	16824352	9.6	8.45	2.22	0.000123	0.198375	XYLT1
62	16687875	10.61	9.46	2.21	0.045004	0.714357	JUN
63	16709072	9.48	8.35	2.19	0.002215	0.397731	ADD3
64	16906419	6.73	5.61	2.18	0.028857	0.658607	SLC40A1
65	17092081	7.91	6.79	2.17	0.004207	0.440384	GLIS3
66	17114272	8	6.89	2.17	0.000034	0.198375	GPC4
67	17106688	8.03	6.92	2.16	0.003064	0.406417	GRIA3
68	17074029	7.38	6.27	2.15	0.010791	0.508075	TDRP
69	16754397	6.29	5.18	2.15	0.004317	0.440384	LOC652993
70	16689546	8.07	6.97	2.15	0.002184	0.396235	TGFBR3
71	16997010	6.03	4.94	2.13	0.017556	0.58068	OTTHUMG00000163317
72	17004989	7.05	5.97	2.12	0.001592	0.376991	RNF182
73	16923766	8.31	7.25	2.08	0.003201	0.413056	COL18A1
74	16832350	9.58	8.54	2.07	0.000392	0.263671	KSR1
75	16781511	5.34	4.29	2.07	0.011554	0.517382	RNA5SP382
76	17020258	5.01	3.97	2.06	0.046083	0.717241	BMP5

77	16687737	10.22	9.18	2.05	0.042364	0.702396	PPAP2B
78	16995989	5.96	4.92	2.04	0.002448	0.404044	FGF10
79	17101292	8.17	7.14	2.04	0.010667	0.508075	STS
80	16696295	9.41	8.39	2.03	0.000133	0.198375	KIFAP3
81	16915530	4.49	3.46	2.03	0.034023	0.672731	MIR548AG2
82	16665588	7.06	6.05	2.02	0.042012	0.702396	ROR1
83	16949759	8.52	9.52	-2.01	0.000136	0.198375	HES1
84	16836021	6.04	7.06	-2.03	0.010652	0.508075	ABCC3
85	16904324	10.66	11.68	-2.03	0.006644	0.469744	FAP
86	16743707	4.44	5.47	-2.04	0.010679	0.508075	MMP10
87	17059119	9.28	10.33	-2.06	0.028998	0.659121	SEMA3C
88	16885290	6.34	7.38	-2.06	0.000553	0.285095	GYPC
89	17084025	5.26	6.32	-2.09	0.047689	0.719821	FLJ35282
90	17003640	7.3	8.36	-2.09	0.001358	0.35533	ADAMTS2
91	16972229	5.13	6.23	-2.15	0.023153	0.625718	ANXA10
92	17072601	6.53	7.64	-2.16	0.003707	0.421757	TRIB1
93	17024746	7.11	8.23	-2.17	0.002144	0.396226	ZBTB2
94	16712292	5.73	6.88	-2.22	0.002665	0.404044	PTPLA
95	16894710	6.38	7.56	-2.27	0.01071	0.508075	FAM49A
96	16856803	6.72	7.95	-2.35	0.011267	0.514555	GADD45B
97	16738630	6.53	7.76	-2.35	0.000374	0.263671	LPXN
98	16927633	7.48	8.73	-2.39	0.018384	0.586973	SDF2L1
99	16818359	7.66	8.91	-2.39	0.002451	0.404044	TGFB1I1
100	16851486	6.43	7.73	-2.46	0.007203	0.472433	LAMA3
101	16843162	6	7.3	-2.47	0.006176	0.462493	EVI2B

102	17063221	6.44	7.75	-2.48	0.001754	0.383976	FAM180A
103	16886717	9.72	11.04	-2.5	0.01795	0.583334	GALNT5
104	16677451	6.04	7.39	-2.55	0.003262	0.413056	KCNK2
105	17049904	4.79	6.15	-2.58	0.02116	0.609685	LRRC17
106	16691090	5.86	7.27	-2.66	0.014476	0.552858	PTPN22
1-7	16819325	9.52	10.94	-2.68	0.007272	0.472433	HERPUD1
108	17020846	8.51	9.95	-2.73	0.02339	0.627571	COL12A1
109	16932483	4.38	5.84	-2.75	0.010589	0.508075	OTTHUMG00000150605
110	16749583	5.53	7	-2.77	0.01259	0.53004	FAR2
111	17072920	8.18	9.65	-2.77	0.007433	0.472433	WISP1
112	16843167	4.15	5.65	-2.84	0.016867	0.573091	EVI2A
1131114	16858137	7.64	9.14	-2.84	0.047828	0.720122	ICAM1
115	16853716	6.51	8.04	-2.89	0.001064	0.346851	LAMA1
116	16901974	4.82	6.41	-3	0.045208	0.715302	IL1A
117	16901986	5.88	7.47	-3.01	0.013368	0.537579	IL1B
118	16665558	4.21	5.84	-3.09	0.00274	0.404044	DLEU2L
1	16762	7.5	9.16	-3.16	0.003526	0.414166	PTHL

19	661						H
20	16773681	9.66	11.32	-3.17	0.038394	0.686809	MEDAG
21	17046135	8.55	10.22	-3.19	0.001125	0.35235	EGFR
22	16766578	5.47	7.17	-3.24	0.016333	0.565871	DDIT3
23	16761212	6.09	7.82	-3.31	0.003101	0.408572	CLEC2B
24	16743148	5.08	7.05	-3.92	0.007367	0.472433	NOX4
25	16903919	5.02	7.05	-4.09	0.025013	0.63692	ERMN
26	16743721	6.09	8.85	-6.77	0.007019	0.472433	MMP1

EXAMPLE 4

Immunomodulation effects of MSC and MSC-NTF

Mesenchymal stem cells (MSCs) have been shown to have considerable immunomodulatory activities. They are currently being tested in clinical trials for the treatment of various diseases owing to their immunosuppressive properties.

The immunomodulatory properties of MSC and MSC-NTF were compared using *in-vitro* assays measuring their effect on T-cells activation by determining the number of CD4 positive cells and by T-cell cytokine production using ELISA assays.

MATERIALS AND METHODS

Peripheral blood mono-nuclear cells (PBMC) were isolated from fresh peripheral blood of healthy volunteers by Ficoll density centrifugation.

MSC-NTF cells were induced to differentiate using the one step protocol described in Example 1. PBMC were co-cultured with either MSC or with MSC-NTF cells in 12-well plates in culture medium containing RPMI and 10% FBS. PBMC were activated using PHA 10µg/ml. Activated PBMC were cultured alone or co-cultured with either MSC or MSC-NTF cells.

After 4 days of co-culture, the non-adherent PBMC were harvested by gentle pipetting and the culture supernatant was collected for cytokine analysis (IL-10, and IFN-γ) by ELISA.

The non-adherent PBMCs were analyzed by flow-cytometry for the levels of CD4 positive T-cells.

RESULTS

The results are summarized in Table 6 herein below.

Table 6

Cell type	INF-γ (pg/ml)	IL-10 (pg/ml)	CD4 (% positives)
Non-activated PBMC	0.0	0.0	
Activated PBMC	765.8	2016.9	26.4
Activated PBMC + MSC	80.41	953.6	16.58
Activated PBMC + MSC-NTF	9.1	602.8	13.8
MSC	0.0	0.0	
MSC-NTF	0.0	0.0	

These results confirm the immunomodulatory effect of MSC-NTF cells and further demonstrate that such an effect is enhanced as compared to non-differentiated MSCs of the same donor. Interferon-gamma and IL-10 secretion by activated PBMC are significantly downregulated by the MSC-NTF cells by 85 and 3.3 fold respectively. Neither MSC nor MSC-NTF cells alone were found to secrete either Interferon-gamma or IL-10.

In addition, MSC-NTF cells led to a reduction of CD4 positive cells to half their number in the control culture in the absence of MSC-NTF cells (from 26.4 to 13.8%).

EXAMPLE 5

Stability of MSC-NTF

To evaluate post-harvest stability of MSC-NTF cells, freshly harvested MSC-NTF cells (the population having been analyzed to ensure that more than 80 % of the cells thereof expressed CD49a) were re-suspended in culture medium, packed in syringes used for administration to patients and incubated at 2-8 °C for up to 4 days. At 24, 48, 72 and 96 hour time points, cells were sampled and counted and viable cell

concentration and viability were assessed. At each time point the cells were also re-seeded and cultured for three additional days in culture medium at 37 °C to evaluate Delayed Onset Cell Death. Recovery of viable cells and viability was established at each time point.

RESULTS

Viability and viable MSC-NTF cell concentration was shown to be maintained for up to 96 hours when packed in syringes as used for administration to patients in clinical trials. Viable cell concentration was practically unchanged for the first 72 hours and only decreased to about 96 % of time 0 after 96 hours in the syringes (Figure 3A). Furthermore incubation of cells for three additional days to evaluate Delayed Onset Cell Death confirmed that the cells maintain stability and viability for at least 72 hours. At 96 hours there is a decline to 86 % of the number of viable cells recovered (Figure 3B). Based on the recovery of viable cells in the syringes and following 3 days in culture, it appears that cell stability is maintained for up to 96 hours. Cells were shown to maintain their characteristic phenotype and neurotrophic factor secretion properties throughout the 96 hours stability period.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

Citation of identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a culture medium as a carrier and an isolated population of differentiated, human bone-marrow derived mesenchymal stem cells (MSCs) that secrete neurotrophic factors, wherein at least 80 % of said differentiated human bone marrow-derived MSCs express cell surface marker CD49a, wherein said population of differentiated MSCs are *ex vivo* generated by differentiating a population of undifferentiated MSCs of a subject in a single differentiating medium comprising basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), heregulin and cAMP for at least two days, wherein more than 95 % of the cells of said population of undifferentiated MSCs express CD73, CD90 and CD105 and lack expression of CD3, CD14, CD19, CD34, CD45, and HLA-DR as determined by flow cytometry, wherein said differentiating is carried out in a single step and wherein said carrier preserves the number of cells in the composition for at least 24 hours.

2. The pharmaceutical composition of claim 1, wherein at least 90 % of said differentiated human bone-marrow derived MSCs express CD49a.

3. The pharmaceutical composition of claim 1, wherein said differentiating medium is devoid of isobutylmethylxanthine (IBMX).

4. The pharmaceutical composition of claim 1, wherein said neurotrophic factors are selected from the group consisting of GDNF, VEGF and HGF.

5. The pharmaceutical composition of claim 1, wherein said culture medium is DMEM.

Date Reçue/Received Date 2020-07-14

FIG. 1

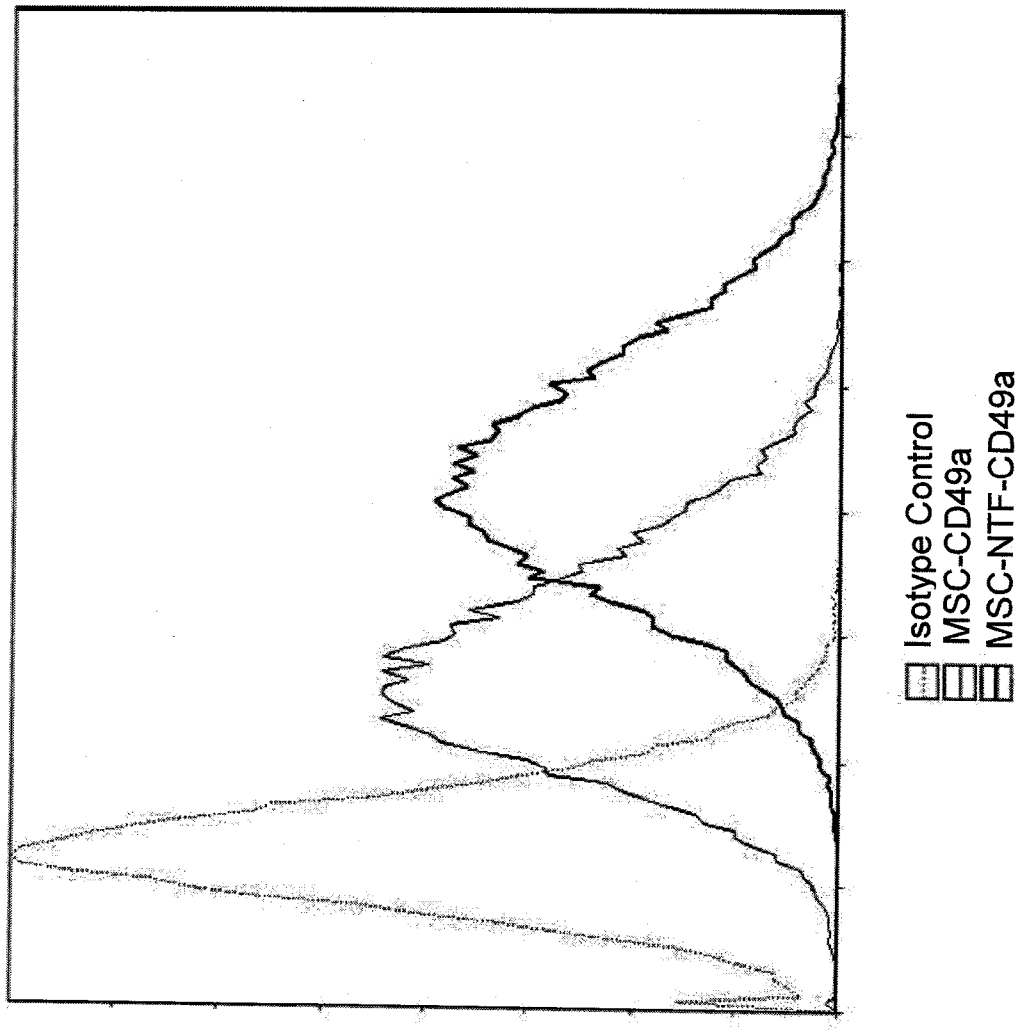


FIG. 2A

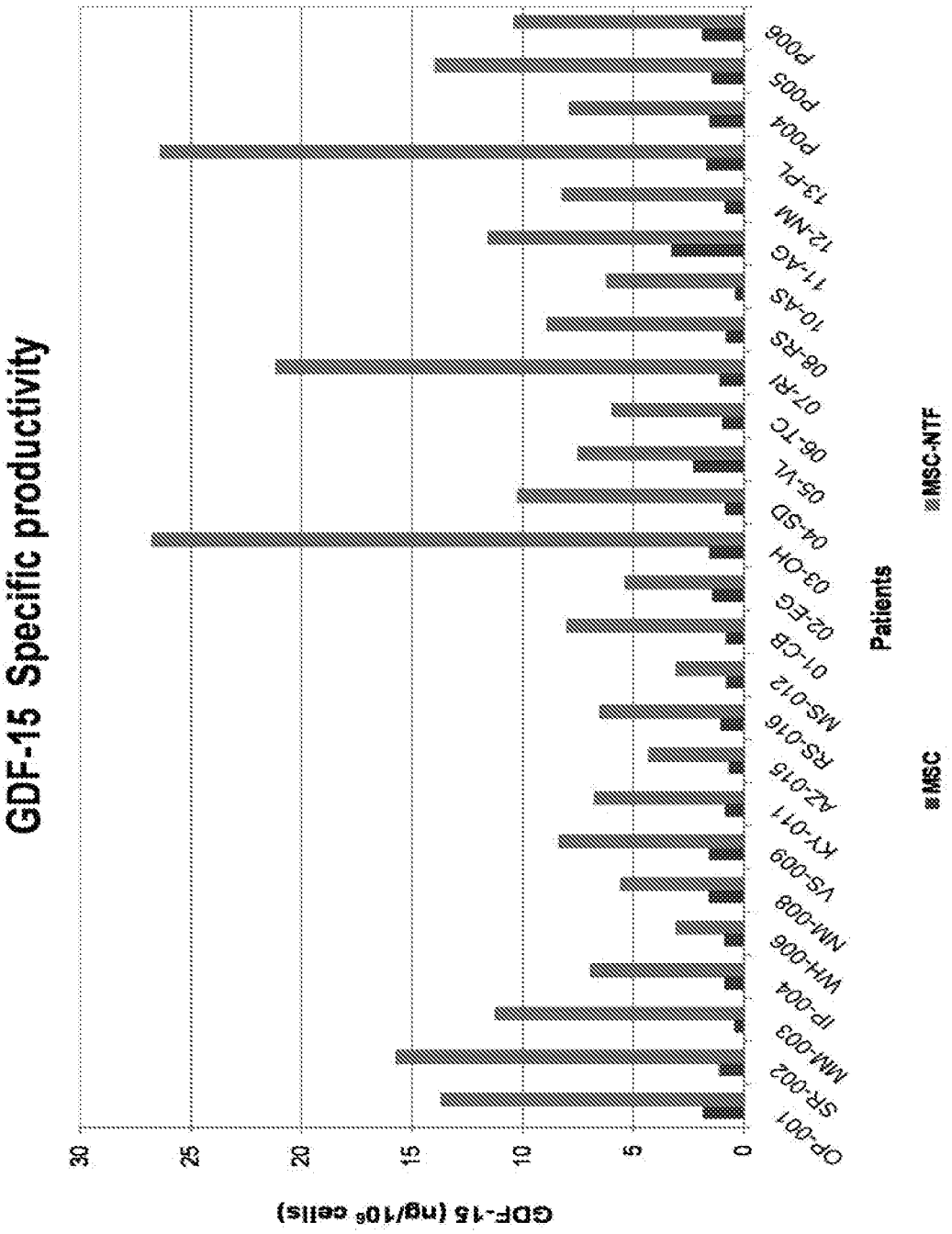

GDF-15 Specific productivity
GDF-15 (ng/10^6 cells)
30
25
20
15
10
5
0
OP-001
SR-002
MM-003
IP-004
WH-006
NM-008
VS-009
KY-011
AZ-015
RS-016
MS-012
01-CB
02-EG
03-OH
04-SD
05-VL
06-TC
07-RI
08-RS
10-AS
11-AG
12-NM
13-PL
P004
P005
P006
Patients
MSC
MSC-NTF

FIG. 2B

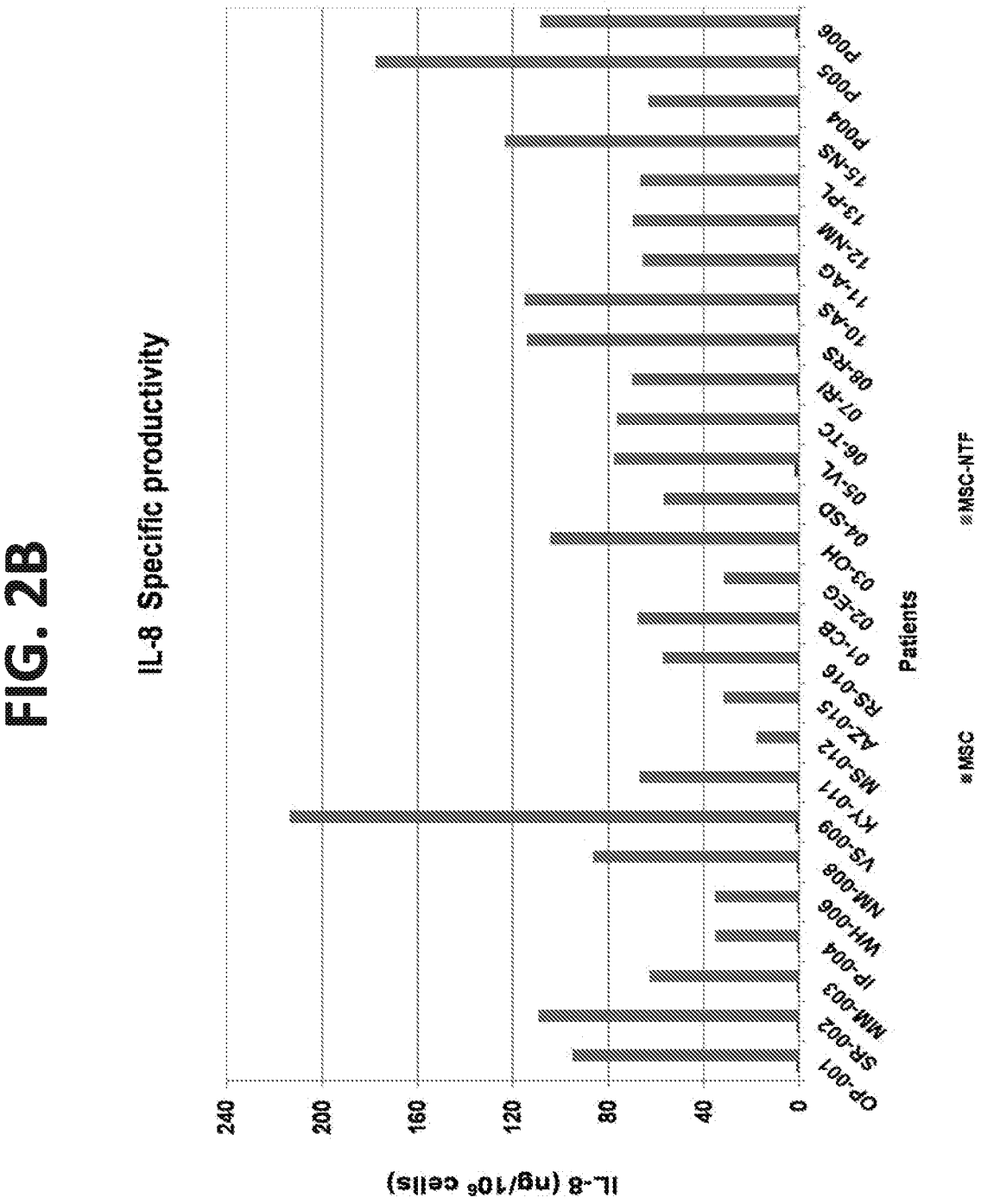

IL-8 Specific productivity
IL-8 (ng/10^6 cells)
240
200
160
120
80
40
0
Patients
OP-001
SR-002
MM-003
IP-004
WH-006
NM-008
VS-009
KY-011
MS-012
AZ-015
RS-016
01-CB
02-EG
03-OH
04-SD
05-VL
06-TC
07-RI
08-RS
10-AS
11-AG
12-NM
13-PL
15-NS
P004
P005
P006
MSC
MSC-NTF

FIG. 3B

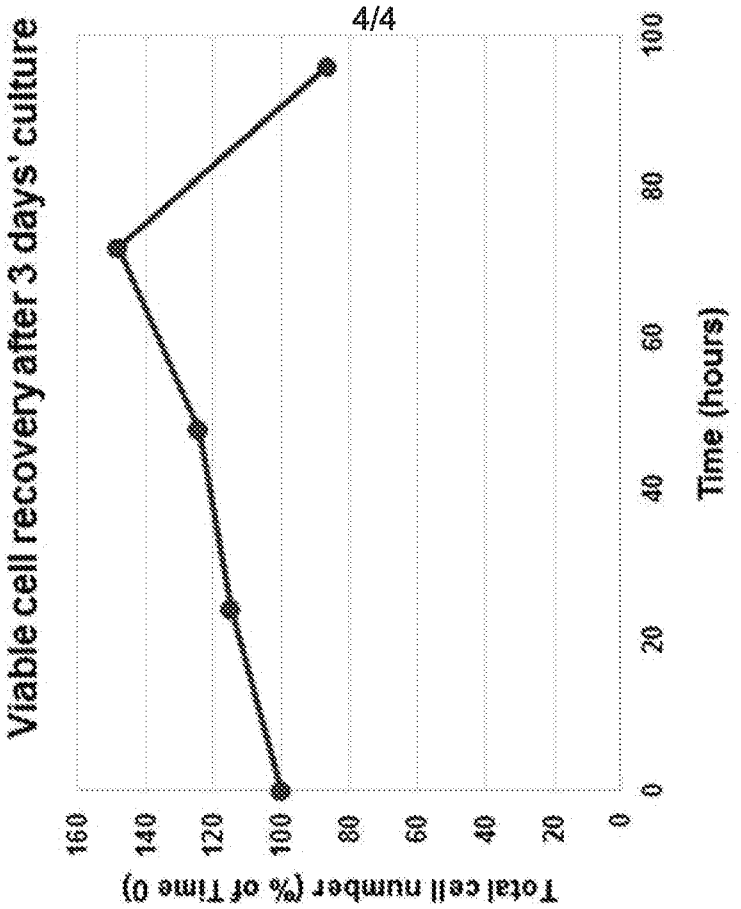
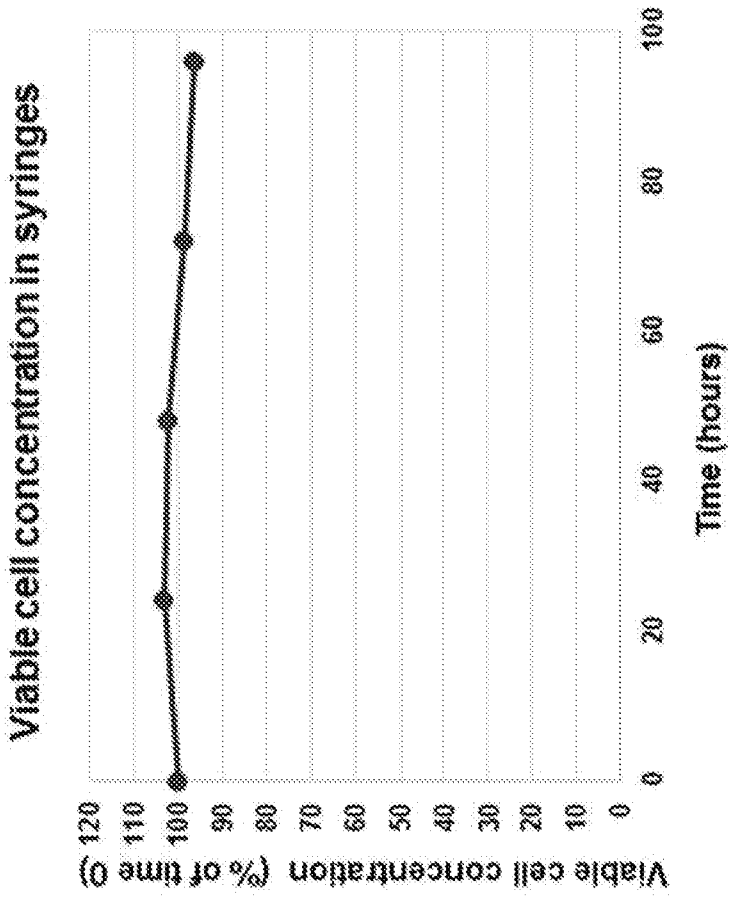
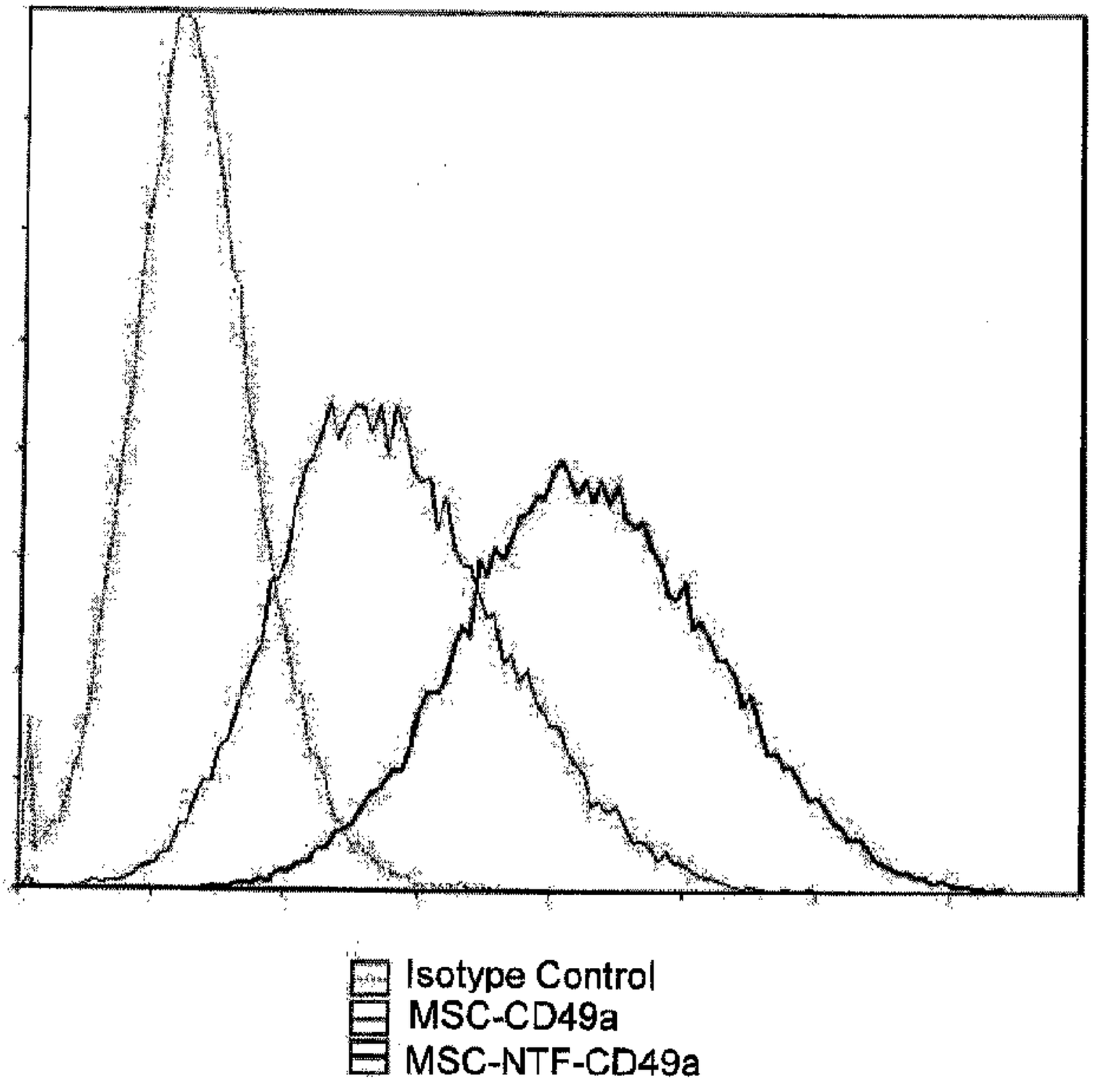

Viable cell recovery after 3 days' culture
4/4
Total cell number (% of Time 0)
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
Time (hours)

FIG 3A


Viable cell concentration in syringes
Viable cell concentration (% of time 0)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
Time (hours)


Isotype Control
MSC-CD49a
MSC-NTF-CD49a