

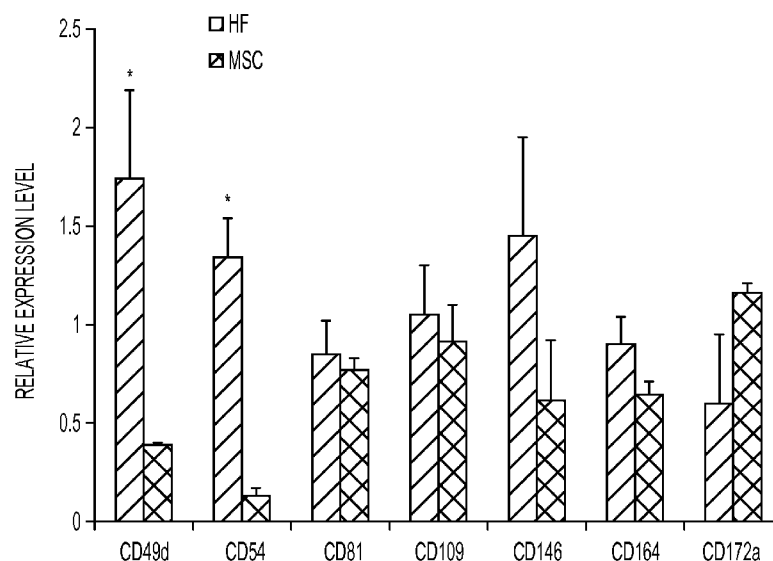
(51) International Patent Classification:
C12N 5/0735 (2010.01)(21) International Application Number:
PCT/US2014/012097(22) International Filing Date:
17 January 2014 (17.01.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
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Street, New York, NY 10007 (US).(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).**Published:**— without international search report and to be republished
upon receipt of that report (Rule 48.2(g))

(54) Title: ENHANCED DIFFERENTIATION OF MESENCHYMAL STEM CELLS

(57) Abstract: Isolated populations of mesenchymal stem cells (MSCs) are provided, including mammalian mesenchymal stem cells (MSCs) characterized by the lack of CD54 (CD54⁻) or low cell-surface CD54 (CD54^{low}). Methods of *in vitro* cell differentiation and methods of treatment using said isolated populations are also provided.

ENHANCED DIFFERENTIATION OF MESENCHYMAL STEM CELLS

FIELD

[0001] This invention generally relates to mesenchymal stem cells, and compositions and methods thereof. U.S. Application Serial No. 12/532,512, filed September 22, 2009, also generally relates to mesenchymal stem cells, the content of which is hereby incorporated by reference in its entirety.

INCORPORATION BY REFERENCE

[0002] This application claims priority to United States Provisional Patent Application No. 61/753,993, filed January 18, 2013. All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

BACKGROUND OF THE INVENTION

[0003] Mesenchymal stem cells (MSCs) are self-renewing multipotent cells capable of differentiating into several cell lineages including osteoblasts, chondrocytes, and adipocytes [1]. First described by Friedenstein *et al.* [2], MSCs have successfully been isolated from bone marrow [2, 3], adipose [4, 5], peripheral blood [6], umbilical cord blood and matrix [7], fetal blood and liver [8], connective tissue of dermis [6, 9], and skeletal muscle sources [6]. The multi-differentiation potential of MSC raises a clinical interest to employ these cells for regeneration purposes, for example, in osteogenesis imperfecta. MSCs lack major histocompatibility complex class II antigens and have been shown *in vitro* to inhibit the activation and/or function of natural killer cells [10], T cells [11-13], dendritic cells [14-16], and B-cells [17]. These immunomodulatory properties have led to clinical trials to assess their therapeutic potential for graft-versus-host disease after hematopoietic transplantation, type I diabetes, and multiple sclerosis. Due to easy access via liposuction, adipose has become the preferred source of MSCs for therapeutic applications.

[0004] Irrespective of their source, MSC isolation involves several steps including positive selection via the properties of plastic-adherence and colony formation [18]. Although this eliminates contaminants such as blood and immune cells, a heterogeneous starting population and fibroblast contamination represent disadvantages. Fibroblasts are known to undergo senescence and apoptosis in culture, while surviving cells become immortal and potentially tumorigenic [19]. Thus, identification and elimination of fibroblasts from MSC culture could improve MSC yield and differentiation potential and also prevent tumor formation after MSC transplantation.

[0005] However, there are currently no markers which can be used to identify and isolate MSCs. Despite consensus that MSCs are positive for expression of CD73, CD90, and CD105, and negative for expression of hematopoietic cell surface markers CD11a, CD19, CD34, CD45, and HLA-DR [20], expression levels of these markers vary across laboratories due to tissue source or the specific culture conditions used [18]. Perhaps more importantly, fibroblasts also express CD105, CD73, and CD90 on their surface and lack hematopoietic markers [21]. Additionally, fibroblasts and MSCs share an almost identical *in vitro* morphology, rendering useless physical filtration techniques [22].

SUMMARY OF THE INVENTION

[0006] Due to their multi-differentiation potential and immunosuppressive function, mesenchymal stem cells (MSCs) hold great promise in regenerative medicine. Lack of specific selection markers to isolate MSCs renders their use at risk of fibroblast contamination. Thus, more effective strategies to purify MSCs are needed.

[0007] One object of the invention is to provide new cell-surface protein markers that can be used for MSC purification during *in vitro* expansion. For example, adipose-derived MSCs (AMSCs) and dermal fibroblasts were compared using real-time RT-PCR and flow cytometry and CD54 was identified as a novel negative selection marker that enhances MSC differentiation potential. With real-time RT-PCR, it was demonstrated that primary human dermal fibroblasts expressed CD54 mRNA 10-fold more than early passage human adipose-derived MSCs (AMSCs). Flow cytometry illustrated 88.0% $\pm$ 4.1% of dermal fibroblasts strongly expressed CD54 on their surface with a mean fluorescence intensity ratio of 24.0 $\pm$ 0.0 compared to 11.0% $\pm$ 0.7% and minimal intensity for AMSCs. Evaluation of CD54 sorted AMSCs revealed CD73 expression was 2.2-fold higher in the

CD54⁻ versus CD54⁺ fraction. CD54⁻ AMSCs demonstrated increased adipogenic and osteogenic differentiation potential relative to CD54⁺ AMSCs. Accordingly, CD54 (also known as ICAM1, or intercellular adhesion molecule 1) is a novel selection marker capable of distinguishing MSCs from fibroblasts and thus enhances MSC osteogenic and adipogenic differentiation potential.

[0008] In one aspect, the invention provides a method for enhancing MSC differentiation potential comprising: (1) isolating a population of MSCs that have low or no cell-surface expression of CD54, and (2) differentiating said CD54^{low} or CD54⁻ MSCs such that the percentage of cells differentiated is greater than the percentage of differentiated cells from CD54⁺ MSCs or from a population of MSCs that are unsorted/non-isolated on the basis of CD54 cell-surface expression.

[0009] As used herein, MSCs can be considered to have “low” cell-surface CD54 expression when the mean fluorescence intensity (MFI) level or median fluorescence intensity level of CD54 relative to an isotype-matched negative control is less than 5, 4, 3, 2, or 1. The mean or median fluorescence intensity level can be measured, for example, by flow cytometry. In flow cytometry, for example, a fluorophore labeled antibody specific to CD54 is used to determine the cell-surface expression level of CD54. An isotype-matched negative control antibody is an antibody that is not specific to CD54, but has the same isotype, *i.e.*, IgG subclass, IgM, IgD, IgE, etc., as the anti-CD54 labeled antibody.

[0010] As used herein, enhanced MSC differentiation refers to comparing the percentage of MSCs that differentiate under standard conditions into different cell-type lineages, such as osteoblasts, chondrocytes, or adipocytes. Other non-limiting examples of mesenchymal derived tissues include fibroblasts, neurons, endothelial cells, myoblasts, and any other MSC-derived cells including corneal stroma and dental pulp of deciduous baby teeth. In a general sense, differentiation of parent MSC cell to progeny cells refers to the loss of some degree of multipotency of the differentiated progeny cells as compared to the parent MSC. MSC differentiation is enhanced if the percentage of differentiated cells is increased when using with CD54^{low} or CD54⁻ MSCs as compared to CD54⁺ MSCs or MSCs that are unsorted or unseparated on the basis of cell-surface CD54 expression. In certain embodiments, MSC differentiation is enhanced if the CD54^{low} or CD54⁻ MSCs provide a greater percentage of differentiated cells, such as about a 1.5-fold, 2-fold, 3-fold, 4-fold, or

5-fold greater percentage, as compared to the percentage of differentiated cells from CD54⁺ MSCs or MSCs that are unsorted or unseparated on the basis of cell-surface CD54 expression.

[0011] As used herein, an MSC can be any mesenchymal stem cell, including MSCs isolated or derived from bone marrow [2, 3], adipose [4, 5], peripheral blood [6], umbilical cord blood and matrix [7], fetal blood and liver [8], connective tissue of dermis [6, 9], and skeletal muscle sources [6]. The MSCs can be any mammalian cell, including rodent, bovine, equine, and primate cells. In a preferred embodiment, the MSCs are human MSCs. Human MSCs have been previously characterized to express a number of cell-surface proteins, including CD54, CD9, CD29, CD44, CD56, CD61, CD63, CD71, CD73, CD90, CD97, CD98, CD99, CD105, CD106, CD112, CD146, CD155, CD166, CD276, and CD304. Human MSCs are often characterized by the cell-surface expression of CD29, CD44, CD73, CD90, and CD105, (with a consensus of CD73, CD90, and CD105), the absence of cell-surface expression of hematopoietic markers such as CD11a, CD19, CD34, CD45, and the absence of HLA-DR [20].

[0012] In another aspect, the invention provides an isolated population of MSCs where the population is characterized by low or no cell-surface CD54 expression. In certain embodiments, the isolated population comprises, for example, greater than 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of CD54^{low} or CD54⁻ MSCs.

[0013] In one embodiment, the isolated population of MSCs is characterized by having a cell-surface marker expression profile comprising: CD54^{low} or CD54⁻, CD73⁺, CD90⁺, CD105⁺, CD11a⁻, CD19⁻, and CD34⁻.

[0014] In one embodiment, the isolated population of MSCs is characterized by having a cell-surface marker expression profile comprising: CD54^{low} or CD54⁻, CD29⁺, CD44⁺, CD73⁺, CD90⁺, CD105⁺, CD34⁻, and CD45⁻.

[0015] In one embodiment, the CD54^{low} or CD54⁻ MSCs further express endogenous HLA-G (HLA-G⁺ MSC), HLA-E (HLA-E⁺ MSC), or HLA-G and HLA-E (HLA-G⁺/HLA-E⁺ MSC); wherein such expression may be intracellular, as a cell surface marker(s), and/or as soluble protein. In one embodiment, the isolated MSC population has a cell-surface expression profile comprising CD54^{low} or CD54⁻ and HLA-G⁺. In one embodiment, the CD54^{low} or CD54⁻ MSCs further express indoleamine-pyrrole 2,3, dioxygenase (IDO). In one embodiment, the CD54^{low} or CD54⁻ MSCs further express CD200. Other examples

of MSC cell surface markers may be found in U.S. Application Serial No. 12/532,512, filed September 22, 2009, the content of which is hereby incorporated by reference in its entirety.

[0016] In one embodiment, the invention provides for methods and compositions relating to isolated populations of MSCs that are CD54^{low} or CD54⁻ and are substantially free of fibroblasts. As used herein, substantially free of fibroblasts can be a population of CD54^{low} or CD54⁻ MSCs that comprise less than 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of contaminating fibroblasts.

[0017] In one embodiment, the invention provides an isolated population of MSCs that comprises CD54^{low} or CD54⁻ and cell-surface CD73 expression that is at least 1.5 or 2-fold higher relative to CD54⁺ MSCs. Such a CD54^{low} or CD54⁻ MSC subpopulation that expresses greater amounts of cell-surface CD73 as compared to CD54⁺ MSCs is useful for immunosuppression applications. CD73, a membrane-bound nucleotidase, is pivotal in the conversion of immunostimulatory ATP into adenosine, which exerts potent immunosuppressive effects on both CD4⁺ and CD8⁺ T cells [32, 33]. Thus, increased CD73 expression in CD54⁻ MSCs would benefit their immunosuppressive effects. Accordingly, methods of immunosuppression are also provided by the invention, where the method comprises administering to a subject allogeneic CD54^{low} or CD54⁻ MSCs. For the purposes of this invention, the cells may be allogeneic, autologous, or xenogeneic with respect to the recipient. For example, the CD54^{low} or CD54⁻ MSCs do not require a perfect match of histocompatibility antigens relative to the subject. In one embodiment, the immunosuppression method comprises administering to a subject allogeneic CD54^{low} or CD54⁻ MSCs that express cell-surface CD73 at least 1.3-fold, 1.5-fold, 1.75-fold, or 2-fold greater than allogeneic CD54⁺ MSCs. In one embodiment, the subject is suffering from one or more conditions selected from type 1 diabetes, graft-versus-host disease, autoimmune disease, inflammatory bowel disease, and cardiac disease. In one embodiment, the subject is suffering from a condition selected from the group consisting of graft-versus-host disease, type I diabetes, sclerosis, and lupus. In one embodiment, the subject is suffering from a condition selected from the group consisting of graft-versus-host disease after hematopoietic transplantation, type I diabetes, and multiple sclerosis. In one embodiment, the allogeneic CD54^{low} or CD54⁻ MSCs are administered in an amount sufficient to suppress the activation of CD4⁺ or CD8⁺ T-cells against transplantation, in type I diabetes, or in autoimmune disease.

[0018] In some embodiments, the CD54^{low} or CD54⁻ MSCs described herein are immunosuppressive against the activation and/or function of natural killer cells, neutrophils, T cells, dendritic cells, and/or B-cells. These immunomodulatory properties of the CD54^{low} or CD54⁻ MSCs described herein may be used as therapeutic treatment for graft-versus-host disease after hematopoietic transplantation, type I diabetes, and multiple sclerosis. In one embodiment, CD54^{low} or CD54⁻ MSCs cells described herein may be used in the induction and maintenance of immune system regulation in post-infectious, inflammatory, allergic, autoimmune, allo-immune, vasculitic, degenerative vascular, and graft-versus-host diseases.

BRIEF DESCRIPTION OF THE FIGURES

[0019] A more complete appreciation of the present invention and many of its advantages will be understood by reference to the following detailed description when considered in connection with the following drawings, which are presented for the purpose of illustration only and are not intended to be limiting, and in which:

[0020] **Figure 1.** Expression profile of CD markers on AMSCs and fibroblasts. Cells were taken from early (≤ 5) cell culture passages.

[0021] **Figure 2.** Quantitative evaluation of CD54 protein expression in AMSCs and fibroblasts. (A) Early passage AMSCs and primary human dermal fibroblasts were stained with anti-CD54 specific antibody (black histograms) or an isotype-matched control antibody (grey histograms). (B) Cells stained with anti-CD54 specific antibody or an isotype-matched control antibody was gated on side scatter dot plot versus the FITC profile. (C) Early passage AMSCs were harvested and sorted with anti-CD54 specific antibody by MACS.

[0022] **Figure 3.** Expression of standard MSC markers in CD54⁻ and CD54⁺ AMSC populations. Semi-quantitative PCR was performed using primers specific for CD73, CD90 and CD105.

[0023] **Figure 4.** Colony forming capability of CD54 sorted and unsorted AMSC populations. (A) Cells from unsorted, CD54⁻, and CD54⁺ fractions were seeded at 156 cells/cm³ on 6-well plates in triplicate and cultured for 14 d, then stained with 0.5% crystal violet in 25% methanol. (B) Clone number was counted under light microscopy. * $p < 0.05$.

[0024] **Figure 5.** Adipogenic differentiation of CD54⁻, CD54⁺, and unsorted AMSCs. Unsorted (A and D), CD 54⁻ (B and E), and CD54⁺ (C and F) AMSCs were cultured in adipogenic induction medium (A, B, and C) or normal DMEM medium (D, E, and F), wherein after 14 d, oil red O staining was used to illustrate intracellular lipid droplets.

[0025] **Figure 6.** Quantitative evaluation of adipogenic differentiation of CD54⁻, CD54⁺, and unsorted AMSCs.

[0026] **Figure 7.** Osteogenic differentiation of CD54⁻, CD54⁺, and unsorted AMSCs. CD 54⁺ (A and D), CD54⁻ (B and E), and unsorted (C and F) AMSCs were cultured in osteogenic induction medium (A, B, and C) or normal DMEM medium (D, E, and F), wherein after 21 d, alizarin red staining was used to illustrate calcification of mineralized extracellular matrix formed in the induced cells.

DETAILED DESCRIPTION

[0027] The following explanations of terms and examples are provided to better describe the present compounds, compositions and methods, and to guide those of ordinary skill in the art in the practice of the present disclosure. It is also to be understood that the terminology used in the disclosure is for the purpose of describing particular embodiments and examples only and is not intended to be limiting.

[0028] MSCs can be obtained, for example, from bone marrow or adipose tissue. As stated above, human MSCs have been previously characterized to express a number of cell-surface proteins, including CD54, CD9, CD29, CD44, CD56, CD61, CD63, CD71, CD73, CD90, CD97, CD98, CD99, CD105, CD106, CD112, CD146, CD155, CD166, CD276, and CD304. Human MSCs can be characterized by the cell-surface expression of CD29, CD44, CD73, CD90, and CD105, and the absence of cell-surface expression of hematopoietic markers CD34 and CD45. Human MSCs can also be characterized by the cell-surface expression profile of CD73⁺, CD90⁺, CD105⁺, CD11a⁻, CD19⁻, and CD34⁻.

[0029] Accordingly, an isolated population of MSCs for enhanced differentiation can be characterized by having a cell-surface marker expression profile of: CD54^{low} or CD54⁻, CD73⁺, CD90⁺, CD105⁺, CD11a⁻, CD19⁻, and CD34⁻. In another embodiment, the isolated population of MSCs for enhanced differentiation can be characterized by having a cell-surface marker expression profile of: CD54^{low} or CD54⁻, CD29⁺, CD44⁺, CD73⁺, CD90⁺, CD105⁺, CD34⁻, and CD45⁻.

[0030] The invention provides for a new sub-population of MSCs that lack or have low CD54 cell-surface expression. This sub-population of MSCs allow for enhanced or superior differentiation of MSCs into different cell-lineages and cell-types, including osteoblasts, chondrocytes, and adipocytes.

[0031] As stated previously, MSCs can be obtained from a variety of tissues. In one embodiment, MSCs are obtained from bone marrow using standard procedures known in the art. For example, bone marrow aspirates can be collected from consenting healthy donors. Human MSCs (hMSCs) can be isolated and cultured as previously described [for example, Aggarwal & Pittenger, (2005), "Human mesenchymal stem cells modulate allogeneic immune cell responses." Blood, 105: 1815–1822]. Generally, mononuclear cells are isolated from bone marrow aspirates by gradient centrifugation. Mononuclear cells are then seeded into flasks containing MSC medium, such as Dulbecco's modified Eagle medium (DMEM)-low glucose supplemented with 10 mM L-glutamine and 10% fetal calf serum (FCS). hMSC cultures are then grown at 37°C under a humidified 5% CO₂ atmosphere. Nonadherent cells are removed after 24 hours (h) by washing with PBS–HSA solution. The medium is changed every 4 days and after 2 weeks the cultures should be mostly confluent. hMSCs are recovered using trypsin and re-plated as passage 1 cells. Subsequently cells can be kept in culture for at least up to 8 passages and tested routinely for the presence of MSC-associated surface molecules. Under such conditions, hMSCs maintain their multilineage capacity to differentiate into osteoblasts, chondrocytes, and adipocytes.

[0032] MSCs can also be obtained from adipose tissue. An exemplary protocol is provided in the Examples below. Description relating to MSC markers, MSC cultures, MSC tissue sources and protocols for isolation therefrom, and assays assessing MSC function can also be found in U.S. Application Serial No. 12/532,512, filed September 22, 2009, the content of which is hereby incorporated by reference in its entirety.

[0033] The invention also provides the use of isolated populations of CD54^{low} or CD54[−] MSCs for purposes of immunosuppression. In one embodiment, MSCs that have a cell-surface profile comprising CD54^{low} or CD54[−] and cell-surface CD73 expression that is at least 1.3-fold, 1.5-fold, 1.75-fold, or 2-fold higher relative to CD54⁺ MSCs are useful for immunosuppression applications as such cells may exert potent immunosuppressive effects on both CD4⁺ and CD8⁺ T cells [32, 33].

[0034] With respect to immunosuppression or immunomodulation, the subject is one in need of immunosuppression or immunomodulation and may be suffering from one or more conditions selected from: type 1 diabetes, graft-versus-host disease, autoimmune disease, inflammatory bowel disease, and cardiac disease. In one embodiment, the subject is suffering from a condition selected from the group consisting of graft-versus-host disease, type I diabetes, sclerosis, and lupus. In one embodiment, the subject is suffering from a condition selected from the group consisting of graft-versus-host disease after hematopoietic transplantation, type I diabetes, and multiple sclerosis. In one embodiment, the allogeneic CD54^{low} or CD54⁻ MSCs are administered in an amount sufficient to suppress the activation of CD4⁺ or CD8⁺ T-cells against transplantation, in type I diabetes, or in autoimmune disease.

[0035] Recently, MSCs have been found to be immunosuppressive against natural killer (NK) cells when grown in MLCs (mixed lymphocyte cultures) at a ratio greater than 1:10 MSC to NK cells. Additionally, MSCs have recently been found to play a significant role in dampening neutrophils' respiratory burst cycle through IL-6 secretion, which in turn preserves neutrophil lifespan in critical reserve sites such as the lungs and bone marrow. When cultured with monocytes, MSCs inhibit the differentiation of dendritic cells and further reduce their potential to activate CD4⁺ T-cells through the down-regulation of MHC class II molecules. Furthermore, when co-cultured with mature dendritic cells, MSCs have been found to reduce secretion of the inflammatory cytokines TNF- α , IL-12, and IFN- γ , while promoting the release of immunosuppressive IL-10. *See, Reddy, et al., Mesenchymal stem cells as immunomodulator therapies for immune-mediated systemic dermatoses, Stem Cells & Dev., 2012, 21(3): p. 352-62, the content of which is incorporated by reference in its entirety.* Thus, in some embodiments, the CD54^{low} or CD54⁻ MSCs described herein are immunosuppressive against the activation and/or function of natural killer cells, neutrophils, T cells, dendritic cells, and/or B-cells. These immunomodulatory properties of the CD54^{low} or CD54⁻ MSCs described herein may be used as therapeutic treatment for graft-versus-host disease after hematopoietic transplantation, type I diabetes, and multiple sclerosis. In one embodiment, CD54^{low} or CD54⁻ MSCs cells described herein may be used in the induction and maintenance of immune system regulation in post-infectious, inflammatory, allergic, autoimmune, allo-immune, vasculitic, degenerative vascular, and graft-versus-host diseases.

EXAMPLES

Materials and Methods

Isolation and cultivation of AMSCs

[0036] AMSCs were isolated from lipoaspirate using a modified method as described [23]. Briefly, lipoaspirate was obtained and washed with equal volume of hank's buffered salt solution (HBSS; Invitrogen, Grand Island, NY). After gentle shaking, isolated samples were separated into two phases. The lower phase (containing stem cells, adipocytes, and blood) was washed and enzymatically dissociated with 0.075% collagenase type I (Sigma-Aldrich, St. Louis, MO)/HBSS for 1 h at 37°C with gentle shaking. Collagenase was inactivated by adding a 1:10 volume of fetal bovine serum (FBS) to adipose collagenase mixture.

[0037] The mixture was centrifuged at 400 g for 10 min at 25°C. The cellular pellet was resuspended in red blood cell lysis buffer (eBioscience, San Diego, CA) and incubated at 25°C for 10 min. The pellet was resuspended in washing medium (HBSS with 2.4% FBS) and sequentially passed through 100, 70, and 40 µm mesh filters to remove cell masses. An equal amount of HISTOPAQUE-1077 (Sigma-Aldrich) was added and centrifuged at 400 g for 30 min to separate MSCs. Cells were seeded at $1-2 \times 10^4$ cells/cm³ and grown at 37°C in dulbecco's modified eagle medium-High Glucose (DMEM, Invitrogen) with 10% FBS and 1% penicillin/streptomycin and equilibrated against 5% CO₂ and 95% air.

[0038] Media was changed every 2-3 d. At 70-80% confluency, cells were washed with PBS and then detached with 0.25% trypsin-EDTA (Invitrogen). Cells were centrifuged at 300 g for 5 min. After removing the supernatant, cells were then resuspended in DMEM culture media and seeded at approximately 5×10^3 cells/cm³ counting as passage one. MSCs were harvested at 70-90% confluency for RNA extraction, flow cytometry, or magnetic-activated cell sorting (MACS) at early (≤ 5) passage.

[0039] Human adult dermal fibroblasts (ATCC, Manassas, VA) were cultured in DMEM-high glucose (Invitrogen) with 10% FBS and 1% penicillin/streptomycin at 37°C in an incubator with 5% CO₂. Cells were harvested at 70-90% confluency for mRNA extraction and flow cytometry.

Real-time and semi-quantitative RT-PCR

[0040] Harvested cells were homogenized using cell shredders (Qiagen Inc, Valencia, CA). Lysates were collected and total RNA retrieved using a Qiagen RNA isolation mini kit (Qiagen Inc, Valencia, CA). RNA purity was determined using spectrophotometry at 260/280 nm. cDNA was synthesized from 2 µg of purified total RNA using reverse transcriptase (SuperScript III kit, Invitrogen).

[0041] Real-time PCR was performed using SYBR green PCR core reagents (Applied Biosystems, Foster City, CA) following the manufacturer's protocol on an ABI Fast Real Time PCR 7900 System (Applied Biosystems, Foster City, CA) as previously described [24]. All primers (see Table 1) were designed using the Primer3 program (Whitehead Institute, Cambridge, MA). The PCR protocols involved activation of DNA polymerase followed by 40 cycles of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min. Each sample was analyzed in triplicate. Reactions without template were used as negative controls. β -actin mRNA was used as an internal control. Standard curves were plotted for each target gene and internal control. RNA quantity was expressed relative to the corresponding β -actin mRNA control. Relative expression levels were calculated using the standard curve method recommended by Applied Biosystems.

Table 1. The sequences of primers used in study.

Gene	Direction	Sequence (5'→3')	Fragment Length	Gene Bank Number
CD54	F	GGCTGGAGCTGTTTGAGAAC (SEQ ID NO:1)	249	NM_000201
	R	TCACACTGACTGAGGCCTTG (SEQ ID NO:2)		
CD49d	F	GTTTCCAGAGCCAAATCCA (SEQ ID NO:3)	185	NM_000885
	R	GCCAGCCTTCCACATAACAT (SEQ ID NO:4)		
CD73	F	CGCAACAATGGCACAATTAC (SEQ ID NO:5)	241	NM002526

	R	CTCGACACTTGGTGCAAAGA (SEQ ID NO:6)		
CD81	F	TCATCCTGTTTGCCTGTGAG (SEQ ID NO:7)	270	NM_003756
	R	CCTCCTTGAAGAGGTTGCTG (SEQ ID NO:8)		
CD90	F	CACACATACCGCTCCCGAACC (SEQ ID NO:9)	190	NM_006288
	R	GCTGATGCCCTCACACTTGACC (SEQ ID NO:10)		
CD105	F	TGCCACTGGACACAGGAT AA (SEQ ID NO:11)	205	NM000188
	R	CCTTCGAGACCTGGCTAGTG (SEQ ID NO:12)		
CD109	F	GTCTCCTTCCCACATCCTCA (SEQ ID NO:13)	192	NM_133493
	R	CAGCTTCTTTCCCAAAGTGC (SEQ ID NO:14)		
CD146	F	ACCCTGAATGTCCTCGTGAC (SEQ ID NO:15)	202	NM_006500
	R	TCTCTGTGGAGGTGCTGTTG (SEQ ID NO:16)		
CD164	F	AAGTGGGGAACACGACAGAC (SEQ ID NO:17)	159	NM_001142401
	R	TGAAACTGGCTGCATCAAAG (SEQ ID NO:18)		
CD172a	F	TGGTAGTGCAGCCTTCTGTG (SEQ ID NO:19)	101	NM-080792
	R	GGCATTGGGTCTCGATAAGA (SEQ ID NO:20)		

[0042] Semi-quantitative PCR was performed in a Bio-Rad DNA Engine thermal cycler using the appropriate oligonucleotide primer pairs (see Table 1). 15 μ L of each PCR

product was detected by ethidium bromide gel electrophoresis using a 1% agarose gel. Each sample was tested in triplicate. Data were analyzed using Alpha Innotech's AlphaEase® FC Software: FluorChem HD2 version 6.0.2. Intensities were measured using the spot-densito tool. The relative expression level was taken as a ratio over the expression of the house keeping gene β -actin.

Flow cytometry

[0043] 1×10^5 cells were collected, washed, blocked with 10% goat serum (Abcam, Cambridge, MA) and diluted in PBS containing 3% BSA (Sigma-Aldrich) for 20 min at 4°C. Cells were then incubated with 2 μ g/mL of CD54 antibody (Biolegend, San Diego, CA) for 1 h at 4°C. Isotype-matched control antibody was used at the same concentration as the primary antibody to evaluate non-specific binding. After 3 washes with PBS, cells were incubated with FITC-conjugated goat anti-mouse secondary antibody for 30 min in the dark at 4°C followed by 3 washes with PBS. Cells were resuspended in PBS containing 3% BSA. Propidium iodide (Vector laboratories, Burlingame, CA) was added at a final concentration of 0.02 μ M for live cell gating. Ten-thousand events were acquired with a FACScaliber flow cytometer (Becton Dickinson, Mountain View, CA) and results were analyzed with CellQuest software program (Becton Dickinson). The cutoff level defined by the isotype control antibody was set to less than 1%. The mean fluorescent intensity (MFI) ratio was calculated by dividing the MFI of CD54 antibody by the MFI of the isotype control antibody.

Magnetic-activated cell sorting

[0044] MACS was performed according to protocols described by Milenyi Biotech Inc (Auburn, CA). Briefly, 1×10^6 cells were labeled with 2 μ g anti-CD54 antibodies in 100 μ L PBS, incubated for 1 h at 4°C and then washed with PBS. Cells were centrifuged at 300 g for 10 min. The supernatant was aspirated and cells were resuspended in 50 μ L of MACS buffer. 20 μ L of goat anti-mouse IgG1 conjugated microbeads were added to cells and incubated at 4°C for 30 min. Cells were then washed, collected, and resuspended in 500 μ L of buffer. MACS columns attached to the magnetic sorter were first rinsed with 3 mL of buffer and eluent discarded. Cells were then applied to the columns and washed

with 3 mL of buffer 3 times, and eluent collected in a single centrifuge tube labeled as CD54⁻ cells. Columns were then removed from the magnetic sorter and 5 mL of buffer was immediately applied. The eluent was collected in a fresh centrifuge tube and labeled as CD54⁺ cells.

[0045] Sorted cells were cultured in a 6 well plate with 2 mL of DMEM-High Glucose (Invitrogen) with 10% FBS and 1% penicillin/streptomycin. Cells were allowed 1 d to recover before they were harvested for mRNA or subjected to either adipogenic or osteogenic differentiation.

CFU-F assay

[0046] Various adipose-derived MSC (AMSC) lines (sorted and unsorted) were seeded at 156 cells/cm³ on 6 well plates in triplicate, cultured for 14 d, and then stained with 0.5% crystal violet (Sigma-Aldrich) in 25% methanol to evaluate clone number.

MSC differentiation

[0047] Reagents used in adipogenic and osteogenic differentiation assays were purchased from Sigma-Aldrich unless otherwise indicated. AMSCs used in the adipogenesis assay were seeded at 1.5 x 10⁴ cells/cm³. Adipogenic induction medium was comprised of DMEM with 4.5 G/L glucose, 2 mM glutamine, 10% FBS, 1% penicillin/streptomycin (Invitrogen) containing 0.5 M IBMX, 2.5 x 10⁻³ M dexamethasone, 1.7 mM insulin, and 140 mM indomethacin. Adipogenic induction medium was applied 1 d after seeding and replaced every 3 d thereafter. Cells were stained with oil red O at 14 d.

[0048] AMSCs used in the osteogenesis assay were seeded at 2.5 x 10³ cells/cm³. Osteogenic induction medium was comprised of DMEM with 4.5 G/L glucose, 2 mM glutamine, 10% FBS, 1% penicillin/streptomycin (Invitrogen) containing 1 M beta-glycerophosphate, 2.5 x 10⁻³ M dexamethasone, and 10 mg/mL ascorbic acid. Osteogenic induction medium was applied 1 d after seeding and replaced every 3 d thereafter. Cells were stained with alazirin red S at 21 d.

Statistical analysis of data

[0049] All data are expressed as means $\pm$ standard error of the mean (SEM) of 3 independent experiments. Analysis of significance was performed using a 2-tailed student t-test with $p < 0.05$ considered significant.

Results

Expression of CD54 marker in AMSCs and fibroblasts

[0050] *In vitro* expanded human AMSCs displayed typical morphology. RT-PCR data demonstrated AMSCs were positive for expression of CD73, CD90, and CD105, and negative for expression of CD11a, CD19, CD34, CD45, and HLA-DR (data not shown). As shown in Figure 1, AMSCs and primary human dermal fibroblasts were harvested and total RNA was isolated. Real-time RT-PCR was conducted to examine the expression of CD49d, CD54, CD81, CD109, CD146, CD164, and CD172a. Y-axis refers to expression levels normalized to β -actin. * $p < 0.05$. Figure 1 shows there was no statistical difference in the relative expression levels of CD81, CD109, CD146, CD164, and CD172a in AMSCs versus fibroblasts whereas fibroblasts expressed significantly higher CD49d and 10.3-fold greater levels of CD54. Thus, we selected CD54 for further analysis.

[0051] Flow cytometry data illustrated that $88.0\% \pm 4.1\%$ fibroblasts strongly express CD54 on the cell surface with a MFI ratio of 24.0 ± 0.0 while only $11.0\% \pm 0.7\%$ of AMSCs showed weak staining (Fig. 2, (A) & (B)). As shown in Figure 2(B), expression of CD54 was analyzed by flow cytometry. MFI of CD54 reactivity normalized to the MFI of the isotype control. Means $\pm$ SEM for 3 independent runs are shown. MACS was used to sort the expanded AMSCs at early passage with anti-CD54 antibody. Consequently we created two new subpopulations, CD54⁻ and CD54⁺ AMSCs. We found $15.8\% \pm 1.9\%$ of AMSCs were CD54⁺ (Fig. 2(C)).

Phenotypic and CFU analysis of CD54⁻ and CD54⁺ AMSCs

[0052] We next isolated RNA from CD54⁻ and CD54⁺ AMSC fractions and analyzed for expression of MSC markers using semi-quantitative RT-PCR. Fifteen μ L of each PCR

product was detected by ethidium bromide gel electrophoresis using a 1% agarose gel. The relative expression level was taken as a ratio over the expression of the house keeping gene β -actin (Figure 3). Figure 3 demonstrates that CD73 mRNA expression was 2.2-fold higher in CD54⁻ versus CD54⁺ cells. Both fractions expressed similar levels of CD90 and CD105.

[0053] Clonogenic assays revealed that early passage unsorted AMSCs displayed the highest colony forming capability with 16 colonies per 1,500 cells seeded (Fig. 4) whereas CD54⁺ and CD54⁻ AMSCs produced 14 and 11 colonies per 1,500 cells seeded, respectively.

Adipogenic and osteogenic differentiation potential of CD54⁻ and CD54⁺ AMSCs

[0054] After 14 d of adipogenic induction, differentiated adipocytes displayed a round shape with the formation of lipid droplets that accumulated the oil red O stain (Fig. 5 A-C). Control cells were negative for oil red O staining (Fig 5. D-F). CD 54⁻ AMSCs showed the highest accumulation of oil red O with 46.7% $\pm$ 5.0% positive staining. Histological staining of lipid droplets was analyzed under light microscopy. The percentage of oil red O stained cells was calculated as the number of oil red O positive cells divided by the total number of cells as indicated by hematoxylin nuclear staining in 3 different fields. * $p < 0.05$ (Figure 6). Positive staining was observed in 28.7% $\pm$ 6.4% of unsorted AMSCs and 16.2% $\pm$ 6.7% of CD54⁺ AMSCs (Fig 6).

[0055] Osteogenic differentiation potential was assessed next. After 21 d of induction, CD54⁻ and unsorted AMSCs stained with alizarin red showed a denser extracellular matrix than did CD54⁺ cells (Fig. 7).

Discussion

[0056] MSCs hold great promise in regenerative medicine but excitement has been tempered due to the possibility of fibroblast contamination [25]. Distinguishing MSCs and fibroblasts is currently not possible as both adhere to plastic and express similar levels of CD73, CD90 and CD105. The aim of our study was to identify a novel selection marker capable of separating MSCs from fibroblasts resulting in enhanced MSC multipotency.

[0057] We focused on CD markers because their cell surface localization allowed for their potential use in sorting procedures. Previous studies from several groups reported qualitative differences in expression of CD49d, CD54, CD81, CD109, CD146, CD164, and CD172a by MSCs and fibroblasts [6, 21, 26-28], but none of these studies confirmed their utility as selection markers. In the present study, mRNA expression levels of these CD markers were further analyzed and validated by real-time PCR, a more quantitative method. We found dermal fibroblasts express 10-fold more CD54 mRNA and 5-fold more CD54 cell surface protein than AMSCs. To our knowledge, this is the first quantitative comparison of CD54 mRNA and protein expression in MSCs and fibroblasts. This finding is in line with a previous report that 70-100% of human adult fibroblasts positively stained with anti-CD54 antibody using flow cytometry [21]. However, a recent study showed that only 25% of human adult dermal fibroblasts were CD54⁺, in line with AMSCs [29]. This discrepancy may be explained by the difference in the experimental design, because endothelial growth medium supplemented with 10% FCS and bFGF was used to culture dermal fibroblasts [29], whereas in our study we used DMEM supplemented with 10% FBS.

[0058] CD54, also called inter-cellular adhesion molecule 1, is a transmembrane glycoprotein with 5 extracellular immunoglobulin G-like domains and a short cytoplasmic tail that associates with multiple cytoskeletal linker proteins [30]. CD54 is primarily expressed in endothelial cells and its interaction with lymphocyte function-associated antigen-1 and macrophage antigen-1 is important for leukocyte adhesion and transendothelial migration [30]. It was reported that lymphocyte function-associated antigen-1-dependent monocyte migration across connective tissue barriers was primarily via engagement of CD54 on fibroblasts [31].

[0059] The difference in CD54 expression by MSCs and fibroblasts suggests that if MSCs become gradually overgrown by contaminating fibroblasts, expression of CD54 should increase at later passage numbers. To test this hypothesis, we compared CD54 mRNA expression in AMSC cultures of passage 2 and 20. The results confirmed our hypothesis as CD54 expression was substantially increased in AMSCs at passage 20 (data not shown). This finding encouraged us to sort AMSCs with anti-CD54 antibody before cell expansion to generate CD54⁺ and CD54⁻ populations. We found CD73 expression was 2.2-fold higher in CD54⁻ cells relative to CD54⁺ cells, while, expression of CD90 and CD105 was similar. CD73, a membrane-bound nucleotidase, is pivotal in the conversion

of immunostimulatory ATP into adenosine, which exerts potent immunosuppressive effects on both CD4⁺ and CD8⁺ T cells [32, 33]. Thus, increased CD73 expression in CD54⁻ AMSCs would benefit their immunosuppressive effects.

[0060] Due to the similarities in morphology and cell surface marker expression, distinction between MSCs and fibroblasts should be based on their differentiation potential. We assessed adipogenic and osteogenic differentiation potential of CD54 sorted and unsorted AMSCs and found that differentiation capacity was enhanced in CD54⁻ AMSCs relative to CD54⁺ cells. Our data suggest CD54 may be utilized to enrich AMSCs for early osteogenic and adipogenic progenitors. Although CD54⁺ cells underwent adipogenic and osteogenic differentiation, the differentiation efficiency was extremely low. The reason why these fibroblast-like cells possess the differentiation capabilities remains unknown. Recently, several papers revealed that fibroblasts derived from skin and/or other sources could be treated to differentiate into several cell types, including osteoblast and adipocyte [9, 29, 34, 35, 36]. Our clonogenic assays illustrated CD54⁻ AMSCs formed slightly less colonies than other populations. This may be due to the fact that the growth rate of fibroblasts (48 h per subculture) is higher than AMSCs (72 h per subculture) under our culture conditions (data not shown). It is also possible that the higher seeding density is disadvantageous in MSC expansion. Such phenomenon was already described [37, 38].

Conclusion

[0061] Herein, we demonstrated that dermal fibroblasts express 10-fold more CD54 mRNA and 5-fold more CD54 protein on their surface than AMSCs. Cultured CD54⁻ AMSCs expressed higher levels of CD73, an immunosuppressive molecule, and increased differentiation capacity into adipocytes and osteoblasts. In conclusion, we identified CD54 as a novel selection marker for distinguishing MSCs from fibroblasts. CD54 may allow for enrichment of MSCs with enhanced multipotency and immunosuppressive properties, both advantageous features for therapeutic applications.

[0062] Those skilled in the art would readily appreciate that all parameters and examples described herein are meant to be exemplary and that actual parameters and examples will depend upon the specific application for which the composition and methods of the present invention are used. Those skilled in the art will recognize, or be able to ascertain

using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that the invention may be practiced otherwise than as specifically described. Accordingly, those skilled in the art would recognize that the use of a composition or method in the examples should not be limited as such. The present invention is directed to each individual composition, or method described herein. In addition, any combination of two or more such compositions or methods, if such composition or methods are not mutually inconsistent, is included within the scope of the present invention.

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We claim:

Claims

1. An isolated population of mesenchymal stem cells (MSCs) comprising mammalian mesenchymal stem cells (MSCs) characterized by the lack of cell-surface CD54 (CD54⁻) or low cell-surface CD54 (CD54^{low}).
2. The isolated population of MSCs of claim 1 comprising greater than 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of CD54^{low} or CD54⁻ MSCs.
3. The isolated population of MSCs of claim 1 that is substantially free of fibroblasts.
4. The isolated population of MSCs of claim 1 having enhanced MSC differentiation.
5. The isolated population of MSCs of claim 4, wherein the CD54⁻ or CD54^{low} MSCs provide a greater percentage of differentiated cells than CD54⁺ MSCs or MSCs that are unsorted or unseparated on the basis of cell-surface CD54 expression.
6. The isolated population of MSCs of claim 4, wherein the CD54⁻ or CD54^{low} MSCs provide a percentage of differentiated cells about 1.5-fold, 2-fold, 3-fold, 4-fold, or 5-fold greater than CD54⁺ MSCs or MSCs that are unsorted or unseparated on the basis of cell-surface CD54 expression.
7. The isolated population of MSCs of claim 1 characterized by having a cell-surface marker expression profile of: CD54^{low} or CD54⁻, CD73⁺, CD90⁺, CD105⁺, CD11a⁻, CD19⁻, and CD34⁻.
8. The isolated population of MSCs of claim 1 characterized by having a cell-surface marker expression profile of: CD54^{low} or CD54⁻, CD29⁺, CD44⁺, CD73⁺, CD90⁺, CD105⁺, CD34⁻, and CD45⁻.
9. The isolated population of MSCs of claim 1, characterized by having cell-surface CD73 expression at least 1.5 or 2-fold higher relative to CD54⁺ MSCs or MSCs that are unsorted or unseparated on the basis of cell-surface CD54 expression.
10. The isolated population of MSCs of claim 1, wherein the CD54^{low} or CD54⁻ MSCs further express cell-surface HLA-G and/or HLA-E.

11. The isolated population of MSCs of claim 1, wherein the CD54^{low} or CD54⁻ MSCs further express cell-surface indoleamine-pyrrole 2,3, dioxygenase (INDO).
12. The isolated population of MSCs of claim 1, wherein the CD54^{low} or CD54⁻ MSCs further express cell-surface CD200.
13. The isolated population of MSCs of claim 1, wherein the MSCs are rodent, bovine, equine, or primate MSCs.
14. The isolated population of MSCs of claim 1, wherein the MSCs are human MSCs.
15. The isolated population of MSCs of claim 1, wherein the MSCs are adipose-derived or bone-marrow derived MSCs.
16. A method for enhancing MSC differentiation potential comprising: (1) isolating a population of MSCs that have low cell-surface expression of CD54 (CD54^{low}) or no cell-surface expression of CD54 (CD54⁻); and (2) differentiating said CD54^{low} or CD54⁻ MSCs such that the percentage of cells differentiated is greater than CD54⁵⁴⁺ MSCs or a population of MSCs that are unsorted or unseparated on the basis of CD54 cell-surface expression.
17. The method of claim 16, wherein the MSCs isolated in step (1) comprise greater than 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of CD54^{low} or CD54⁻ MSCs.
18. The method of claim 17, wherein the MSCs isolated in step (1) comprise less than 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.1% of contaminating fibroblasts.
19. The method of claim 16, wherein the CD54^{low} or CD54⁻ MSCs express cell-surface CD73 at a level at least 1.3-fold, 1.5-fold, 1.75-fold, or 2-fold greater than allogeneic CD54⁺ MSCs or MSCs that are unsorted or unseparated on the basis of cell-surface CD54 expression.
20. The method of claim 16, wherein in step (2) the percentage of cells differentiated is about 1.5-fold, 2-fold, 3-fold, 4-fold, or 5-fold greater than CD54⁵⁴⁺ MSCs or a population of MSCs that are unsorted or unseparated on the basis of CD54 cell-surface expression.

21. The method of claim 16, wherein the MSCs are rodent, bovine, equine, or primate MSCs.
22. The method of claim 16, wherein the MSCs are human MSCs.
23. The method of claim 16, wherein the MSCs are adipose-derived MSCs or bone-marrow derived MSCs.
24. The method of claim 16, wherein in step (2) the differentiated cells belong to the osteoblast, adipocyte, or chondrocyte lineage.

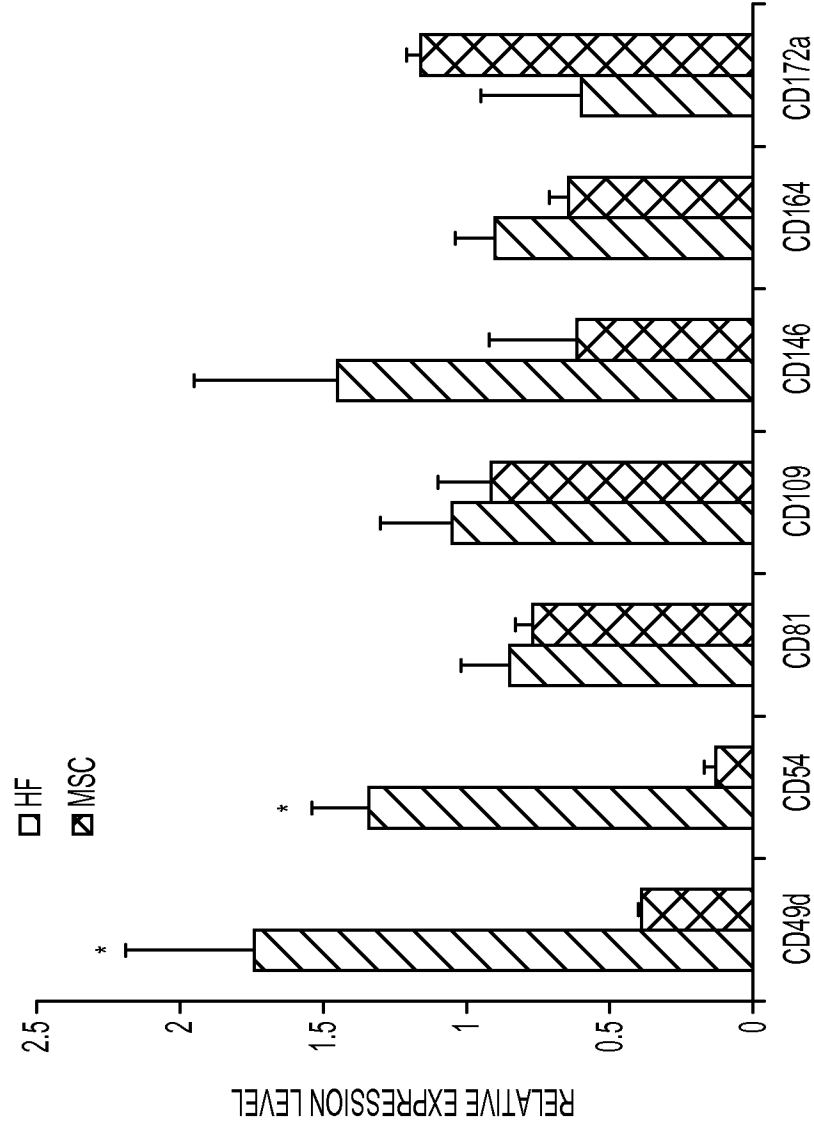


FIG. 1

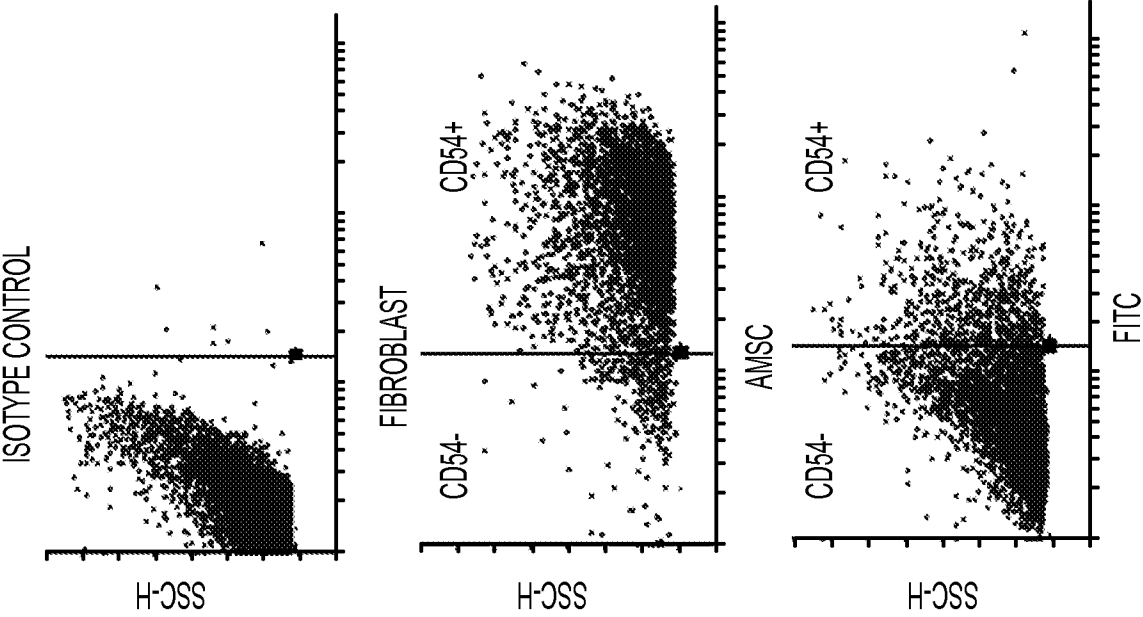


FIG. 2B

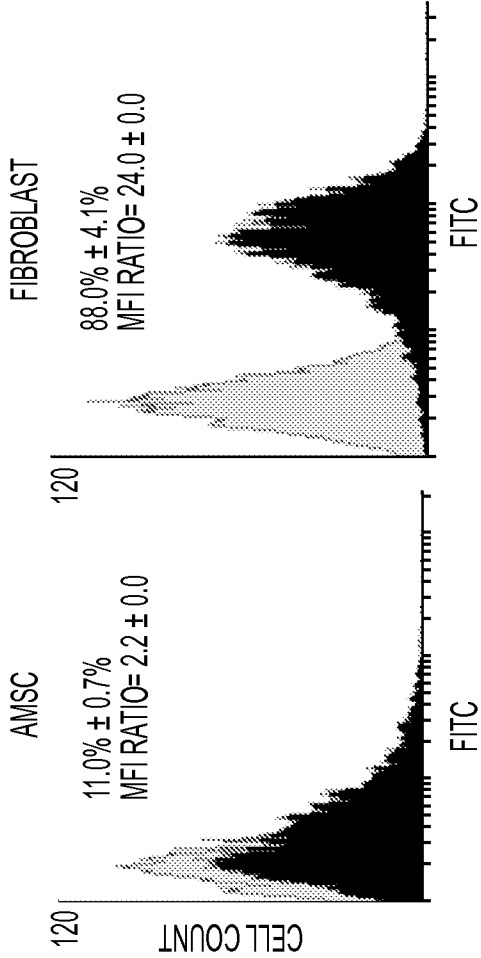


FIG. 2A

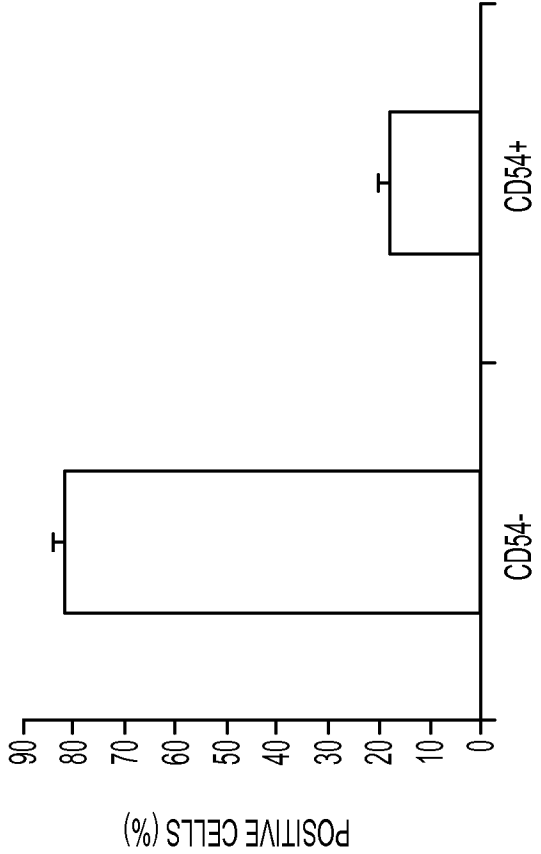


FIG. 2C

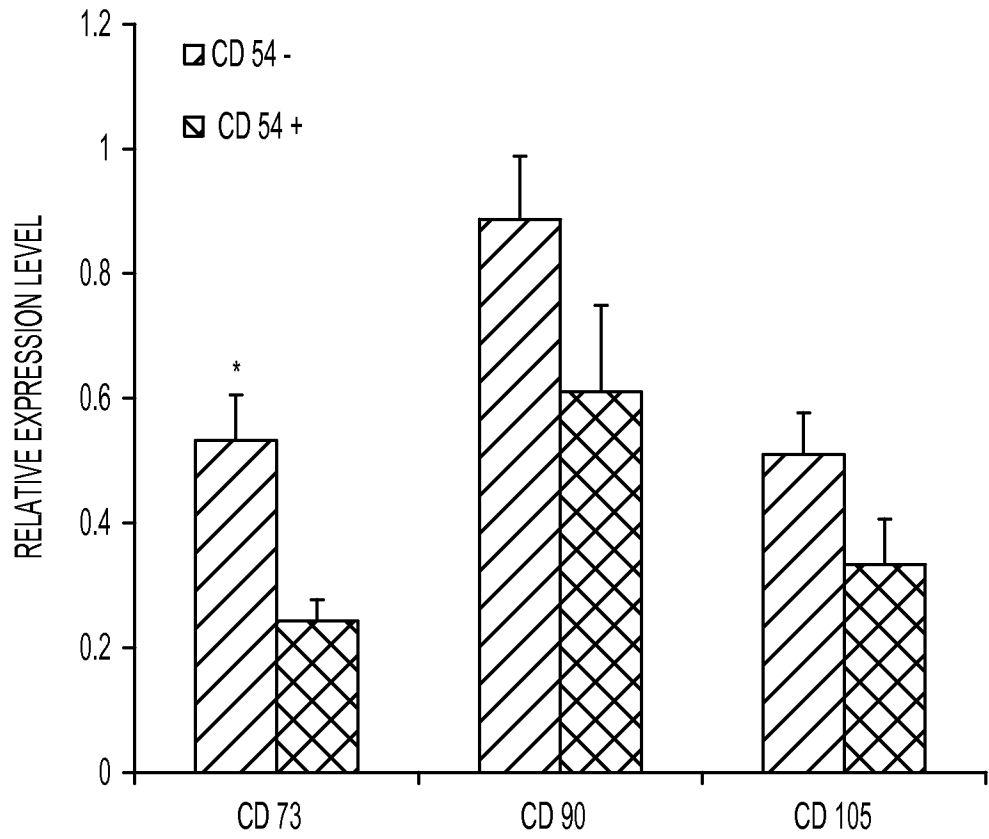


FIG. 3

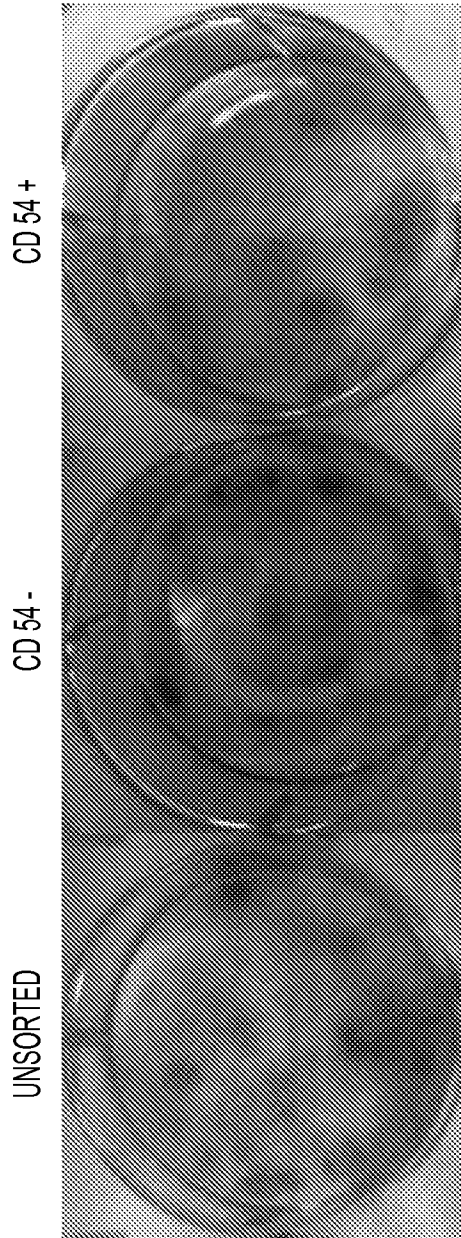


FIG. 4A

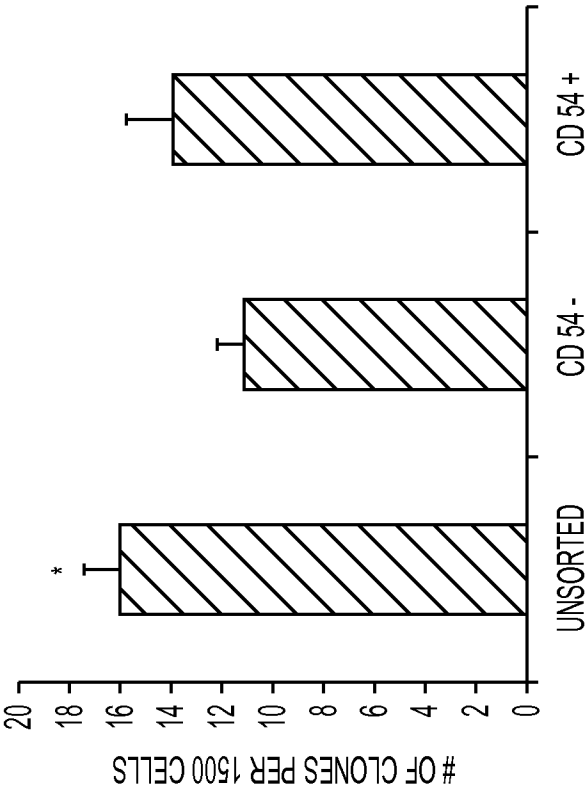


FIG. 4B

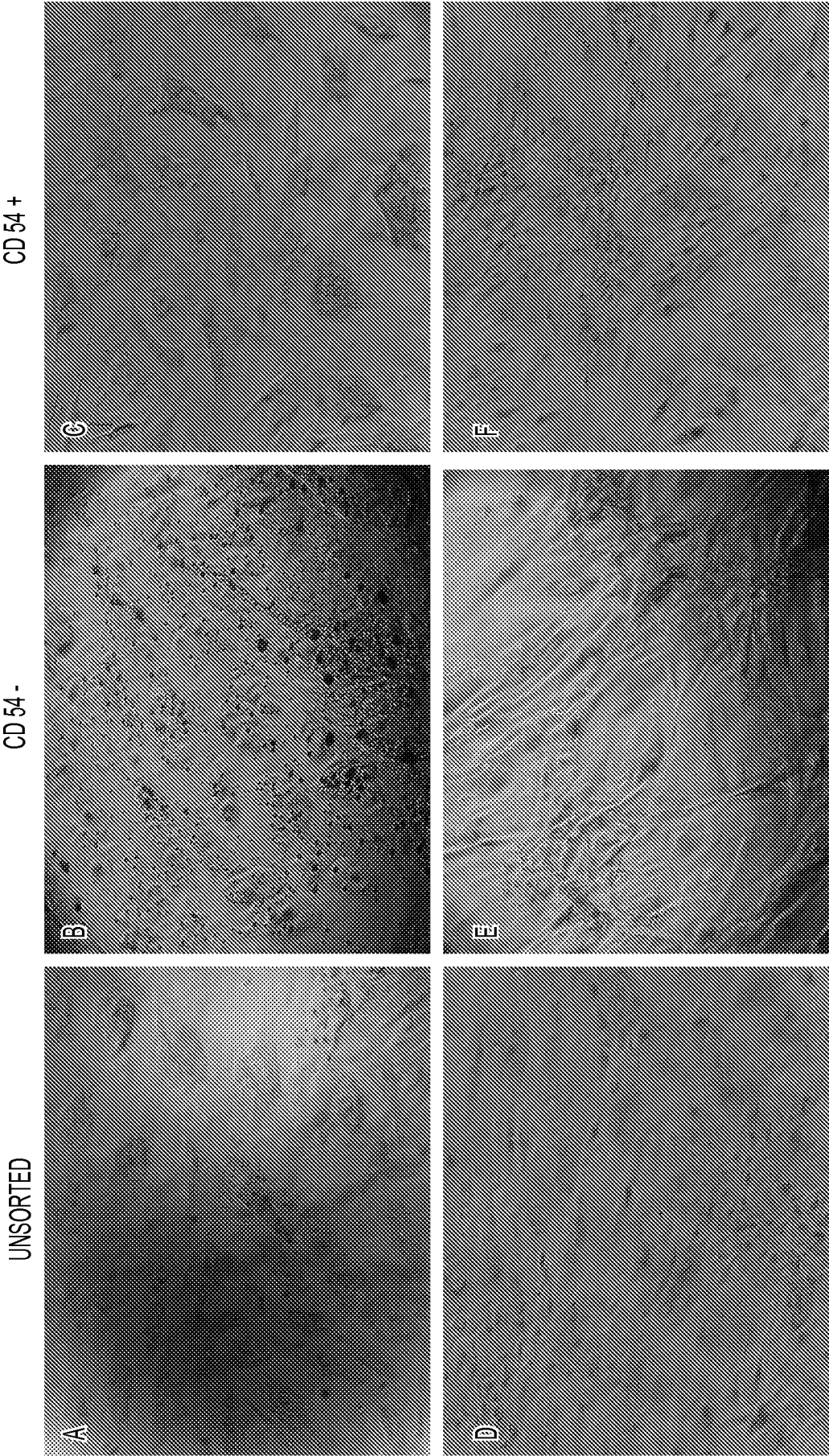


FIG. 5

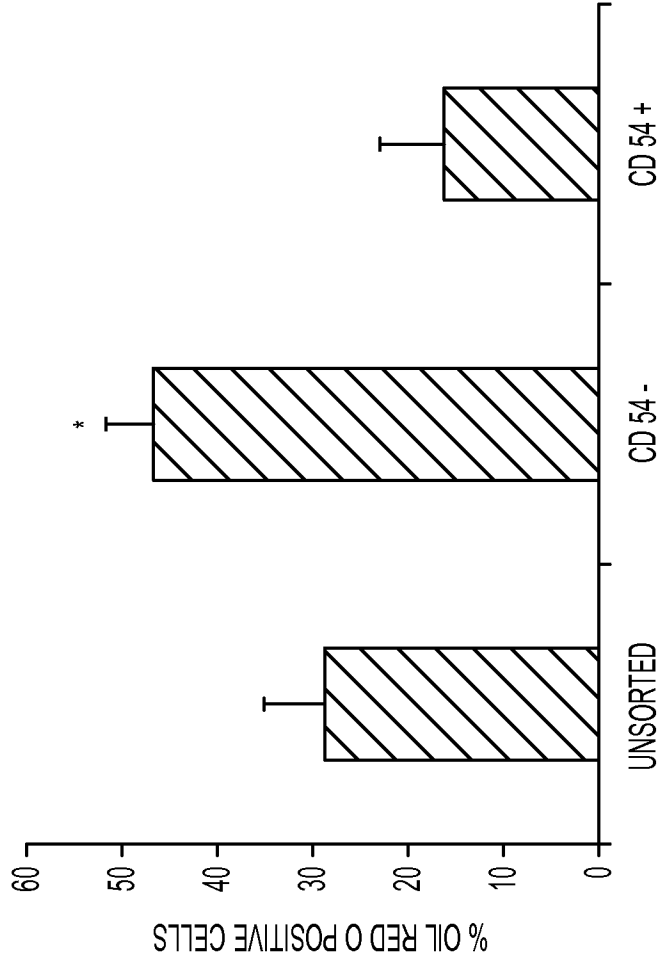


FIG. 6

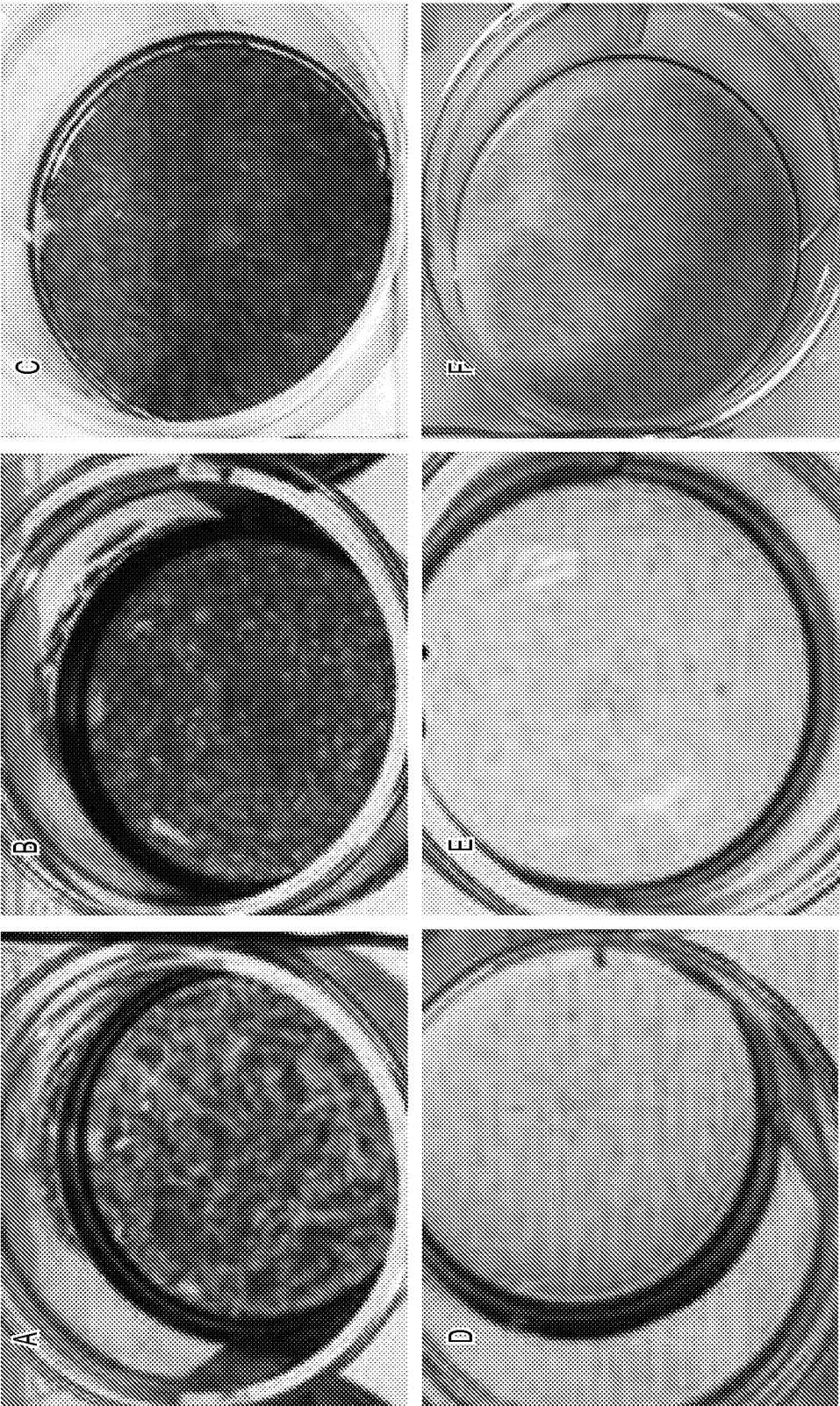


FIG. 7