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## Method for enhancing engraftment of cells using mesenchymal progenitor cells

Beerelli Seshi

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(12) **United States Patent**  
**Seshi**(10) **Patent No.:** **US 7,442,390 B2**  
(45) **Date of Patent:** **Oct. 28, 2008**(54) **METHOD FOR ENHANCING  
ENGRAFTMENT OF CELLS USING  
MESENCHYMAL PROGENITOR CELLS**

WO WO 03/029432 A2 4/2003

(75) Inventor: **Beerelli Seshi**, Torrance, CA (US)

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(73) Assignee: **University of South Florida**, Tampa, FL (US)

( \* ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 362 days.

(21) Appl. No.: **10/887,582**(22) Filed: **Jul. 9, 2004**(65) **Prior Publication Data**

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**Related U.S. Application Data**

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(60) Provisional application No. 60/486,077, filed on Jul. 9, 2003, provisional application No. 60/412,450, filed on Sep. 20, 2002, provisional application No. 60/352,636, filed on Jan. 28, 2002, provisional application No. 60/334,277, filed on Nov. 28, 2001, provisional application No. 60/327,140, filed on Oct. 3, 2001, provisional application No. 60/277,700, filed on Mar. 21, 2001, provisional application No. 60/209,245, filed on Jun. 5, 2000.

(51) **Int. Cl.**  
**A61K 35/26** (2006.01)  
**C12N 5/08** (2006.01)  
**C12N 5/00** (2006.01)(52) **U.S. Cl.** ..... **424/577; 435/372; 435/375**(58) **Field of Classification Search** ..... None  
See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**

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(Continued)

*Primary Examiner*—Michail Belyavskyi(74) *Attorney, Agent, or Firm*—Saliwanchik, Lloyd & Saliwanchik(57) **ABSTRACT**

The present invention provides isolated pluri-differentiated human mesenchymal progenitor cells (MPCs), which simultaneously express a plurality of genes that are markers for multiple cell lineages, wherein the multiple cell lineages comprise at least four different mesenchymal cell lineages (e.g., adipocyte, osteoblast, fibroblast, and muscle cell) and wherein each of the markers is specific for a single cell lineage. The present invention also method for isolating and purifying human mesenchymal progenitor cells from Dexter-type cultures for characterization of and uses, particularly therapeutic uses for such cells. Specifically, isolated MPCs can be used for diagnostic purposes, to enhance the engraftment of hematopoietic progenitor cells, enhance bone marrow transplantation, or aid in the treatment or prevention of graft versus host disease.

**19 Claims, 22 Drawing Sheets**  
**(2 of 22 Drawing Sheet(s) Filed in Color)**

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FIG. 1

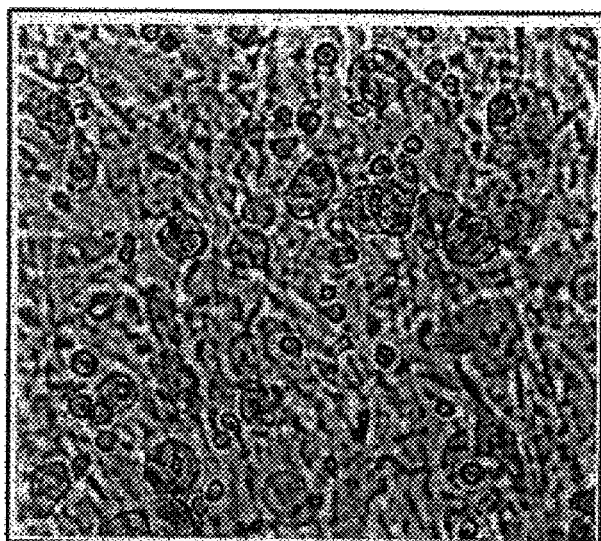


FIG. 2

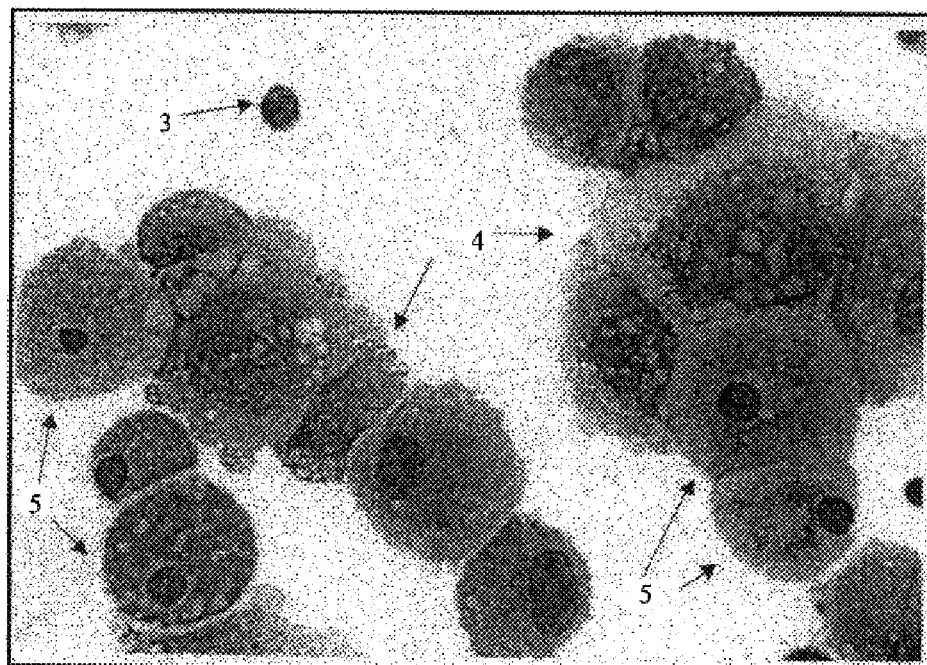
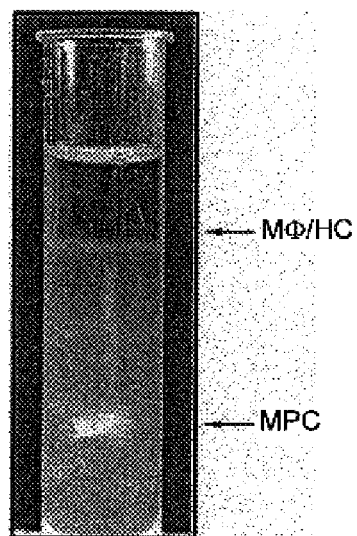
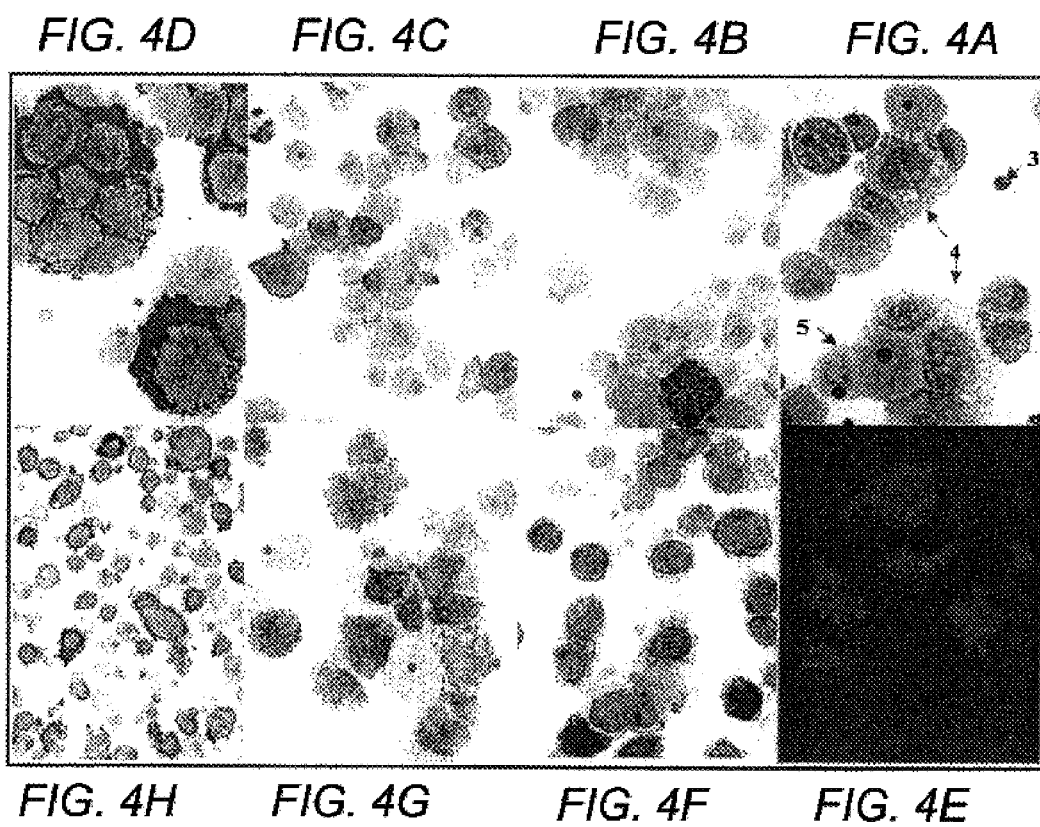
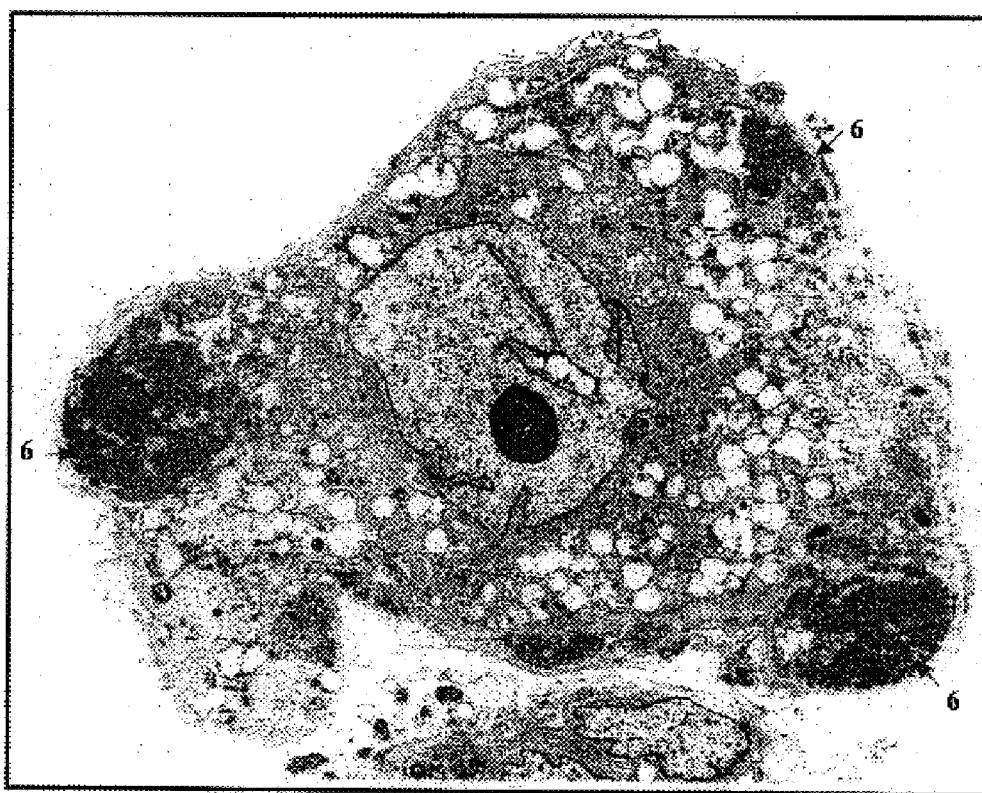
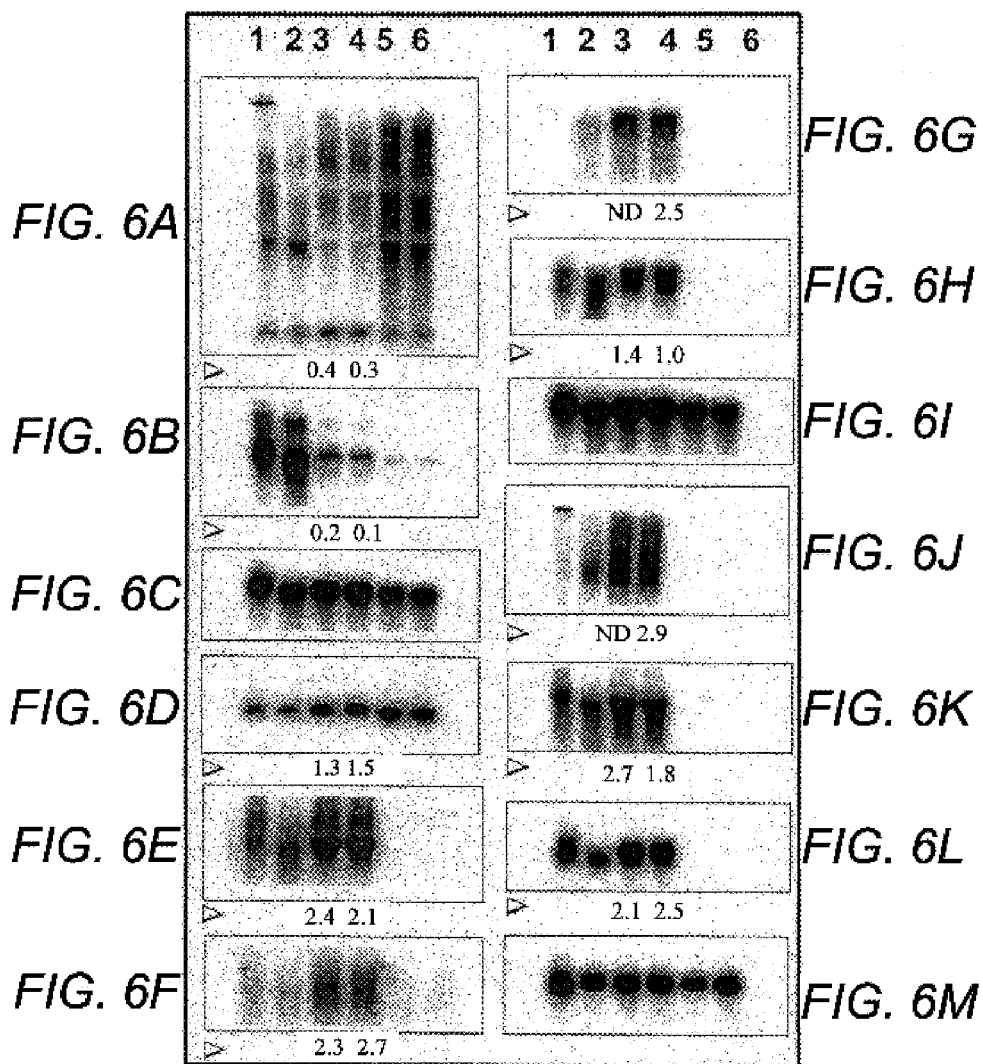


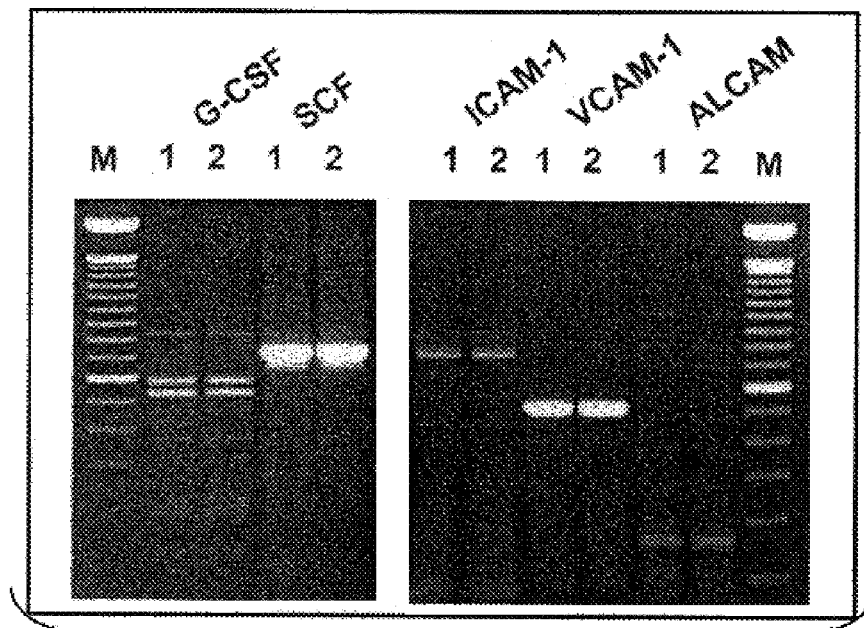
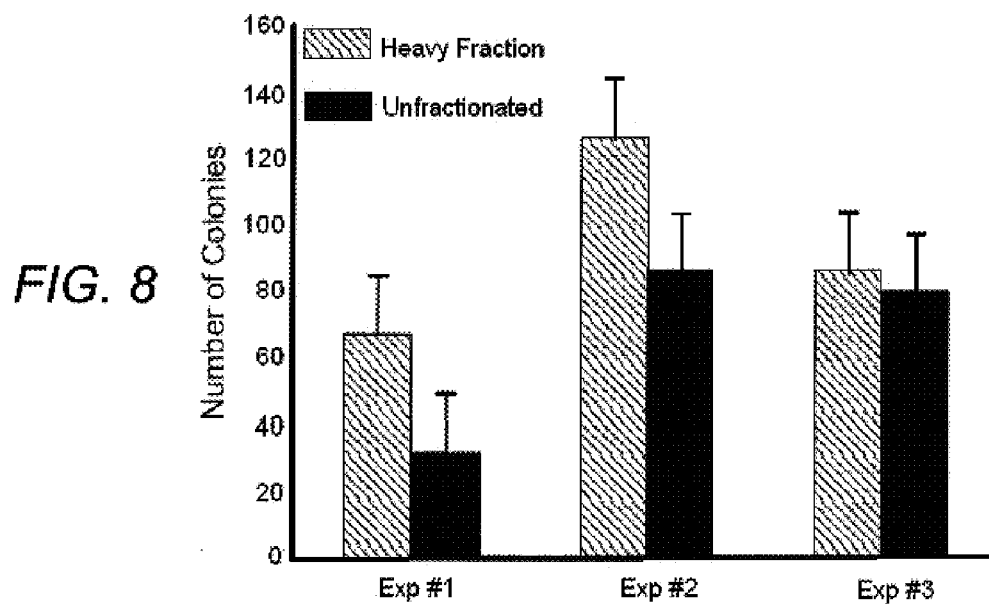
FIG. 3





*FIG. 5*



*FIG. 7**FIG. 8*

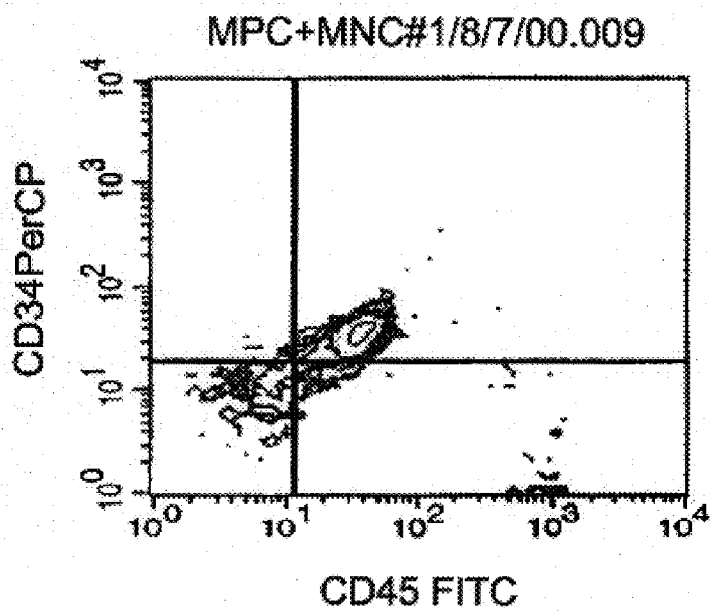
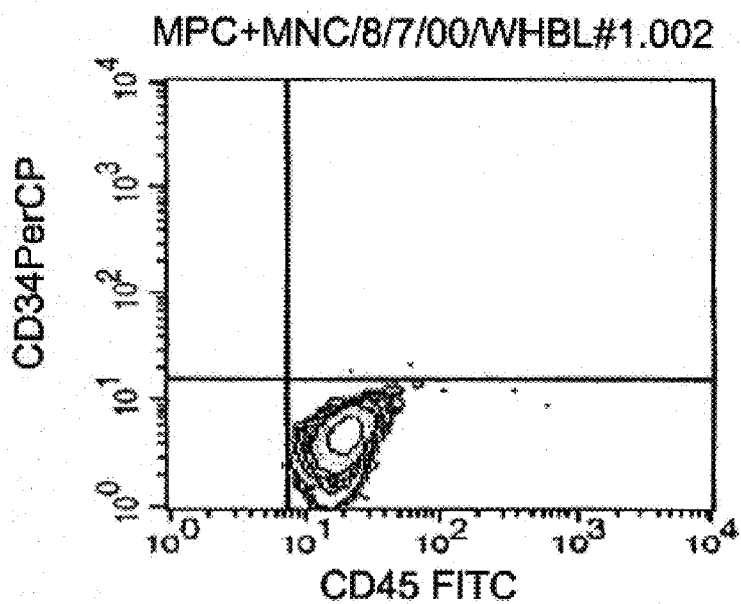
*FIG. 9A**FIG. 9B*

FIG. 10A

FIG. 10C

FIG. 10E

FIG. 10G

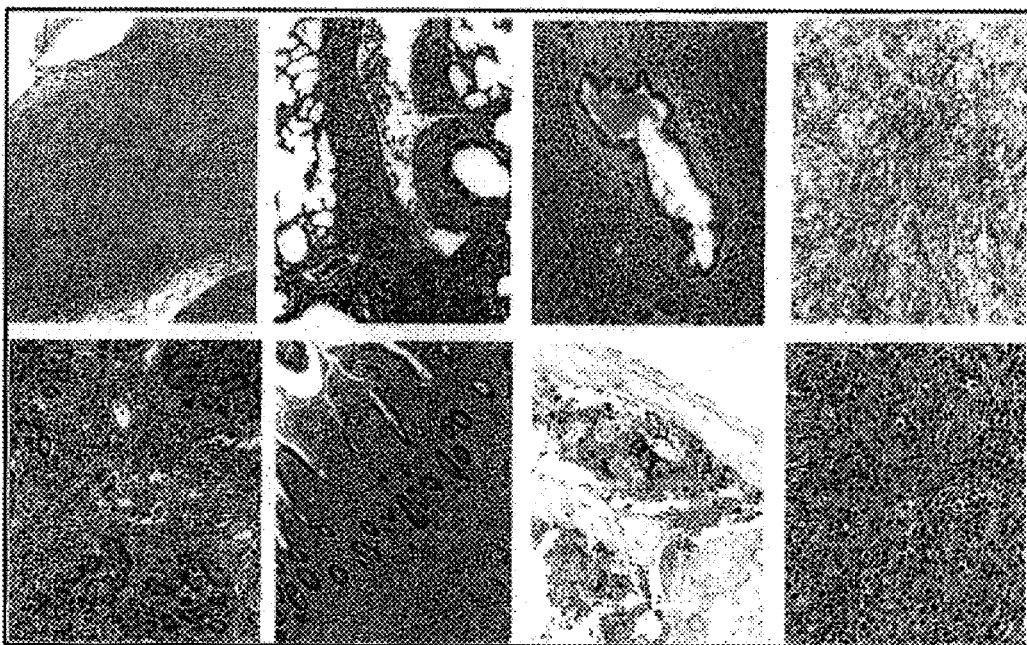


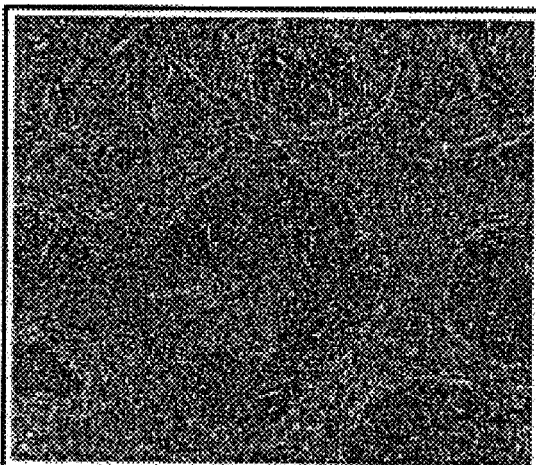
FIG. 10B

FIG. 10D

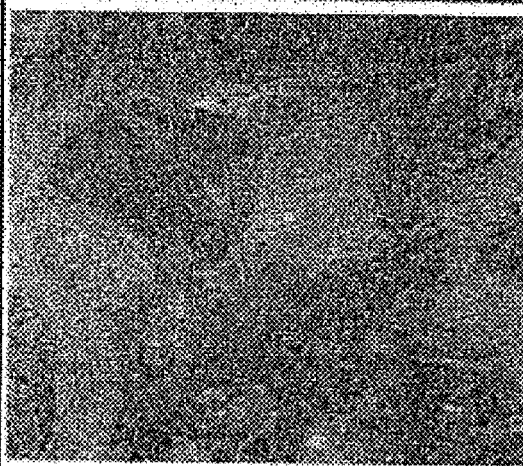
FIG. 10F

FIG. 10H

*FIG. 11A*



*FIG. 11B*



*FIG. 11C*

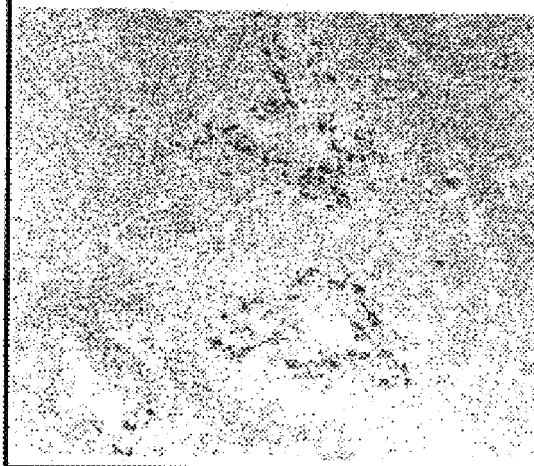




FIG. 12A

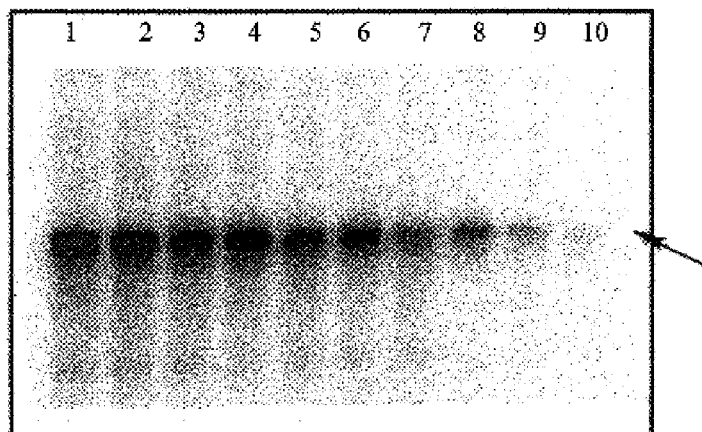


FIG. 12B

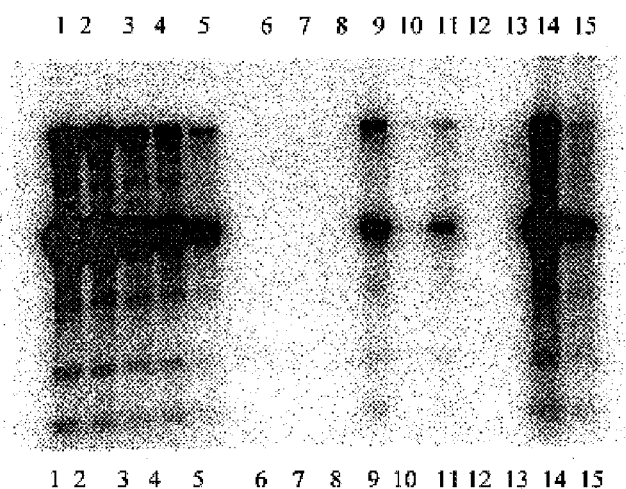
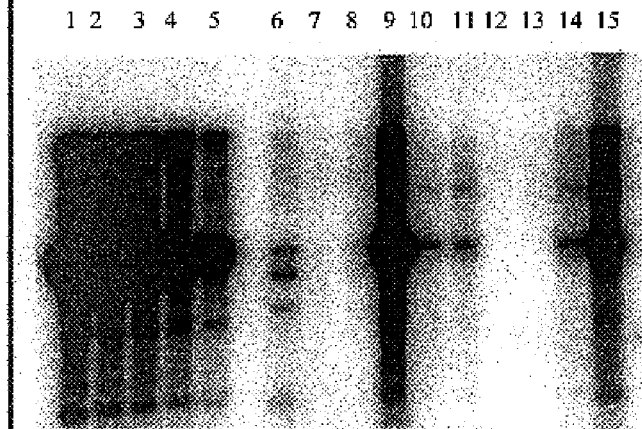
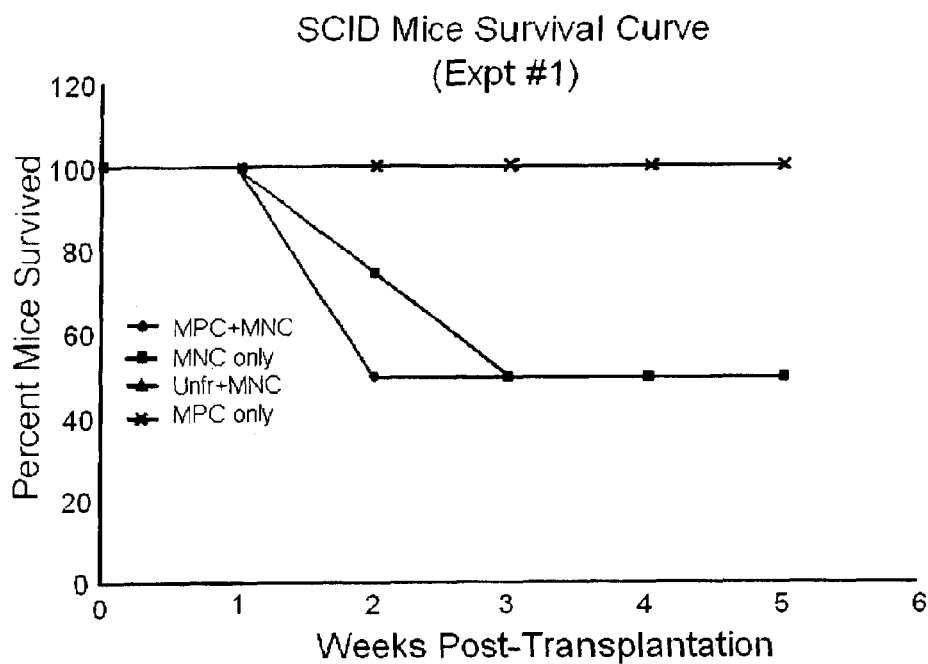
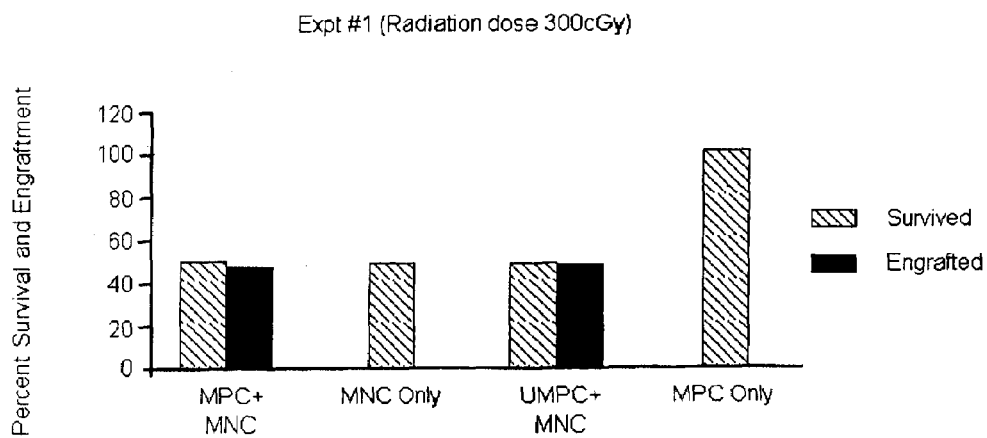
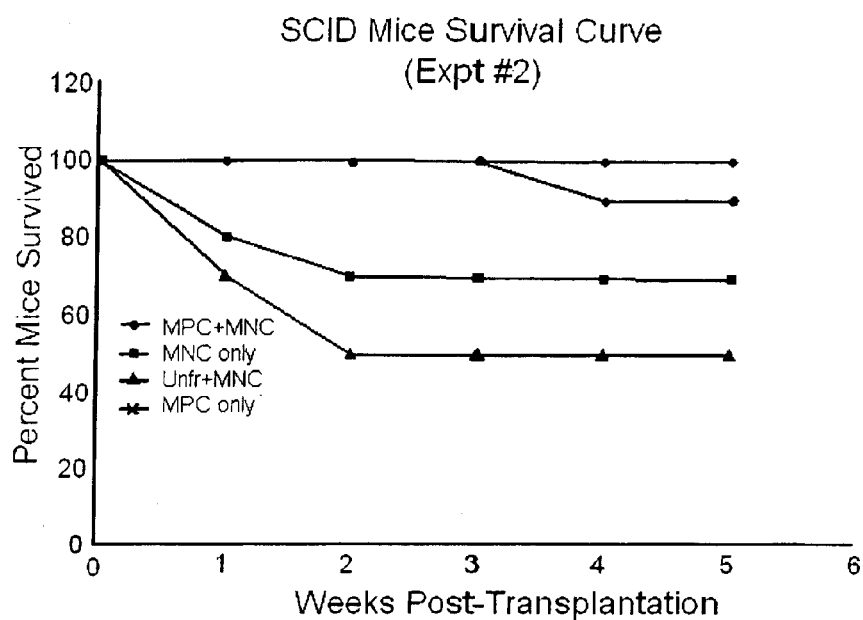
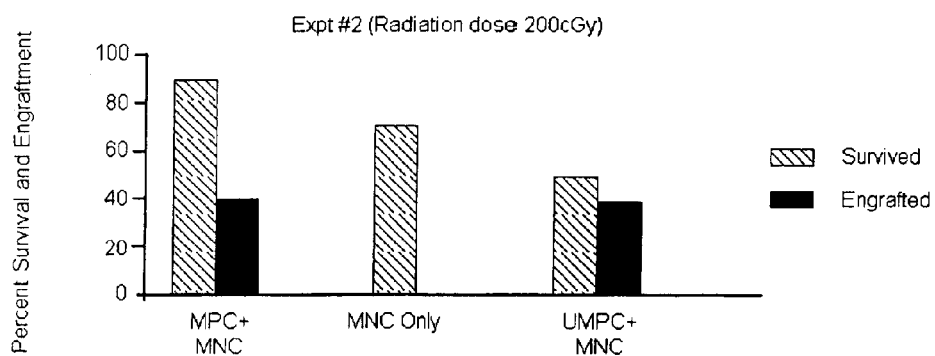
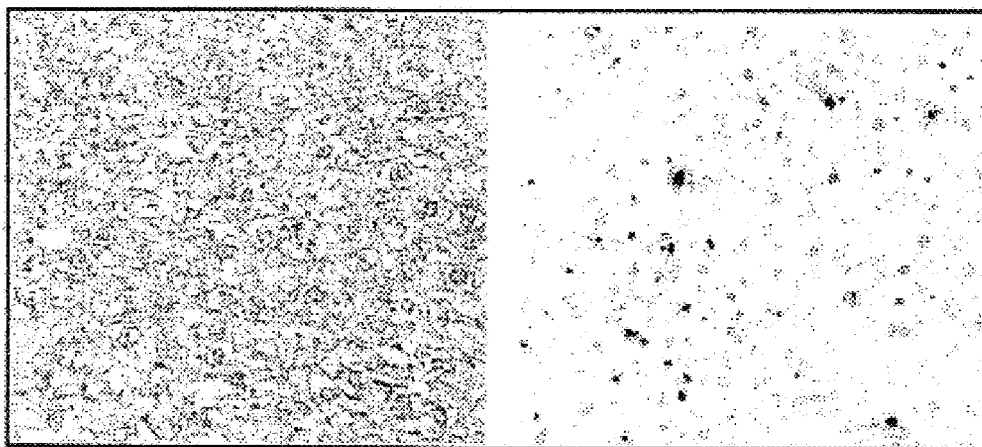


FIG. 12C



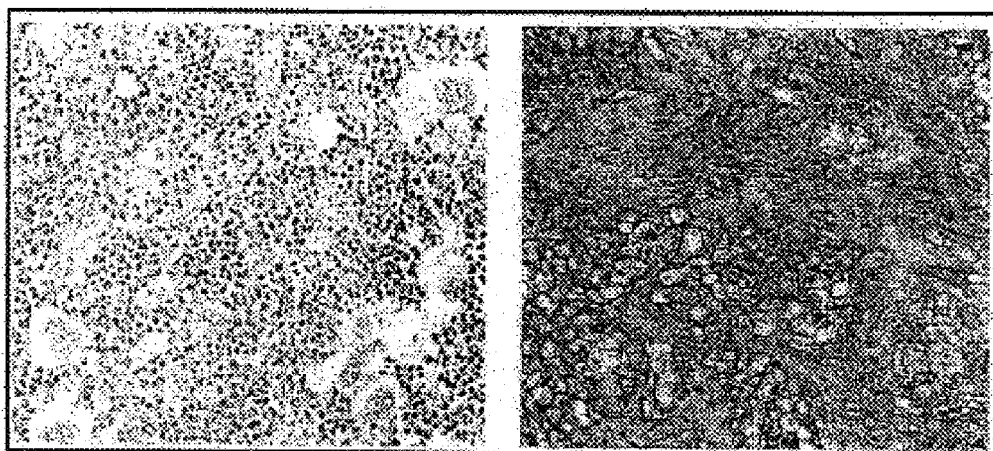
*FIG. 13A-1**FIG. 13A-2*

*FIG. 13B-1**FIG. 13B-2*



*FIG. 14A*

*FIG. 14B*



*FIG. 14C*

*FIG. 14D*

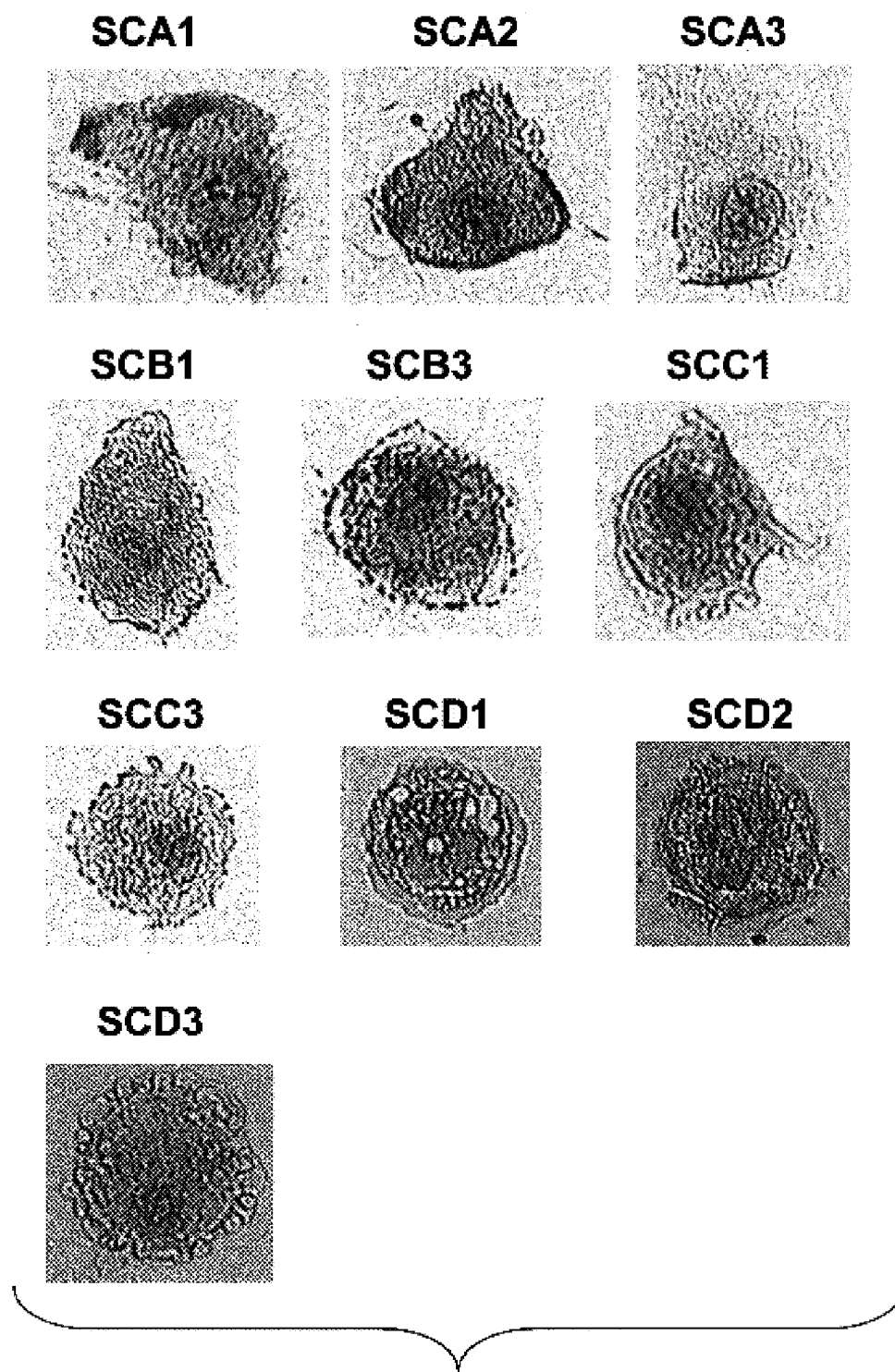


FIG. 15

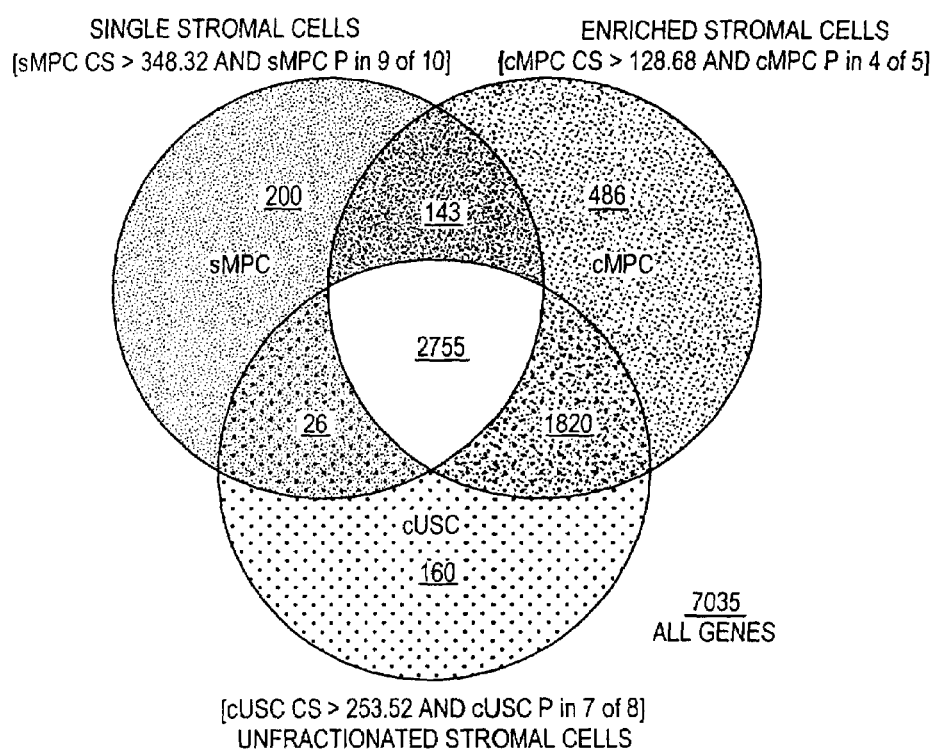


FIG. 16

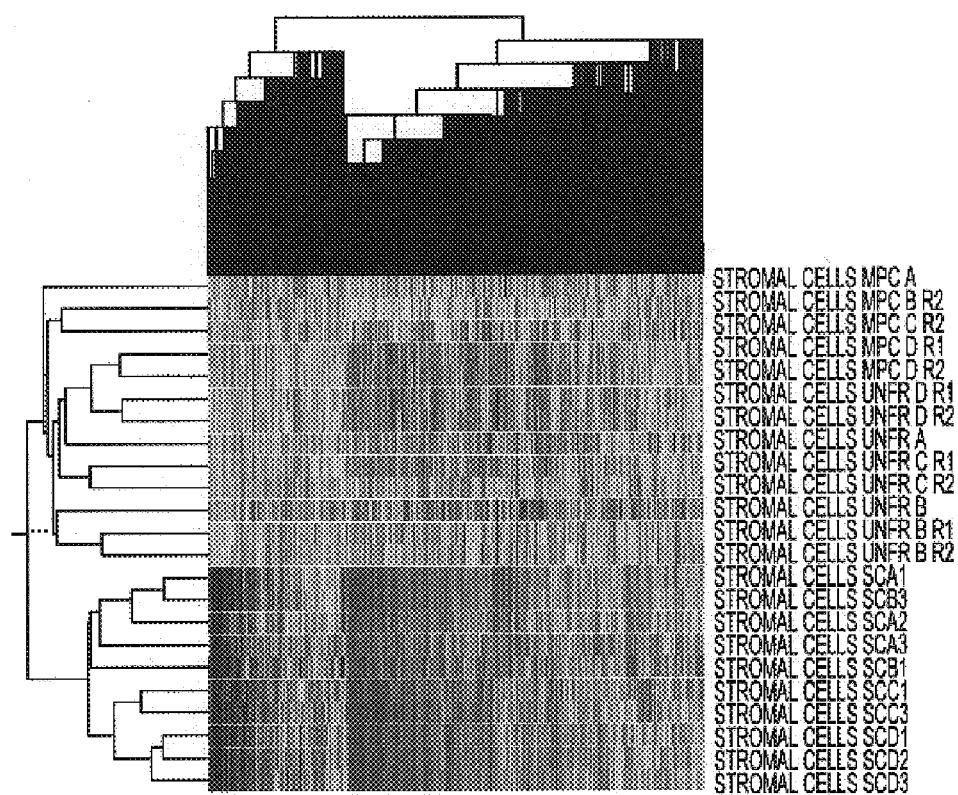


FIG. 17

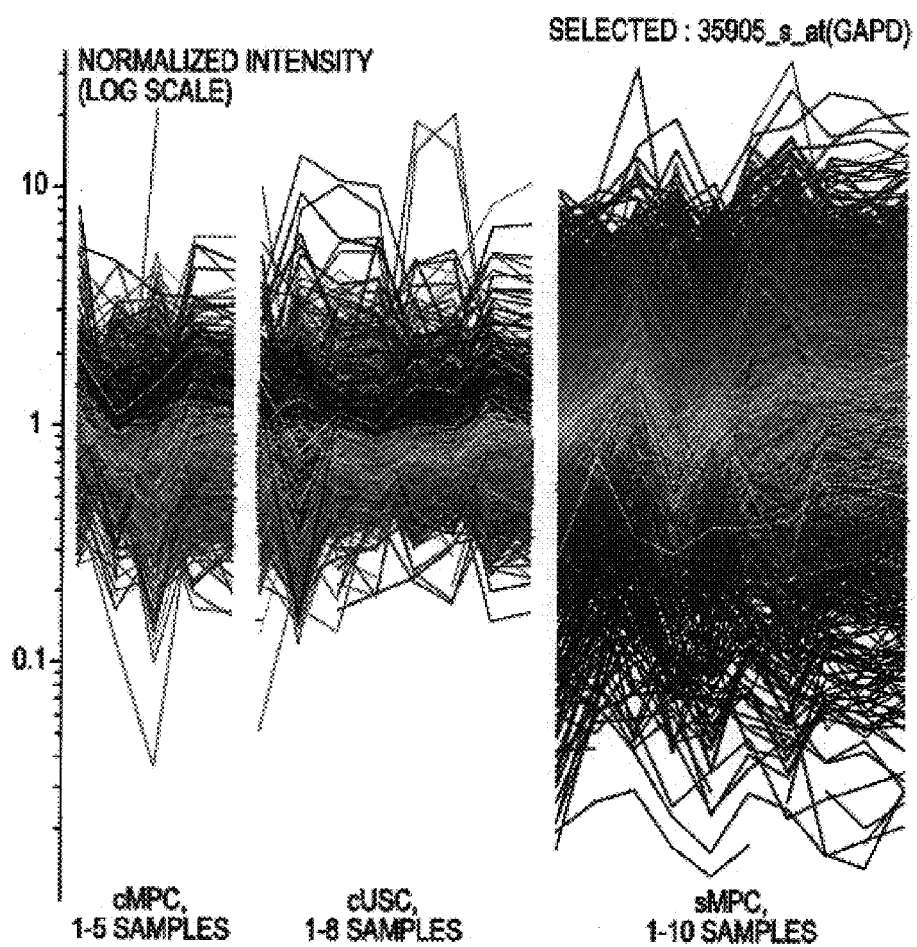
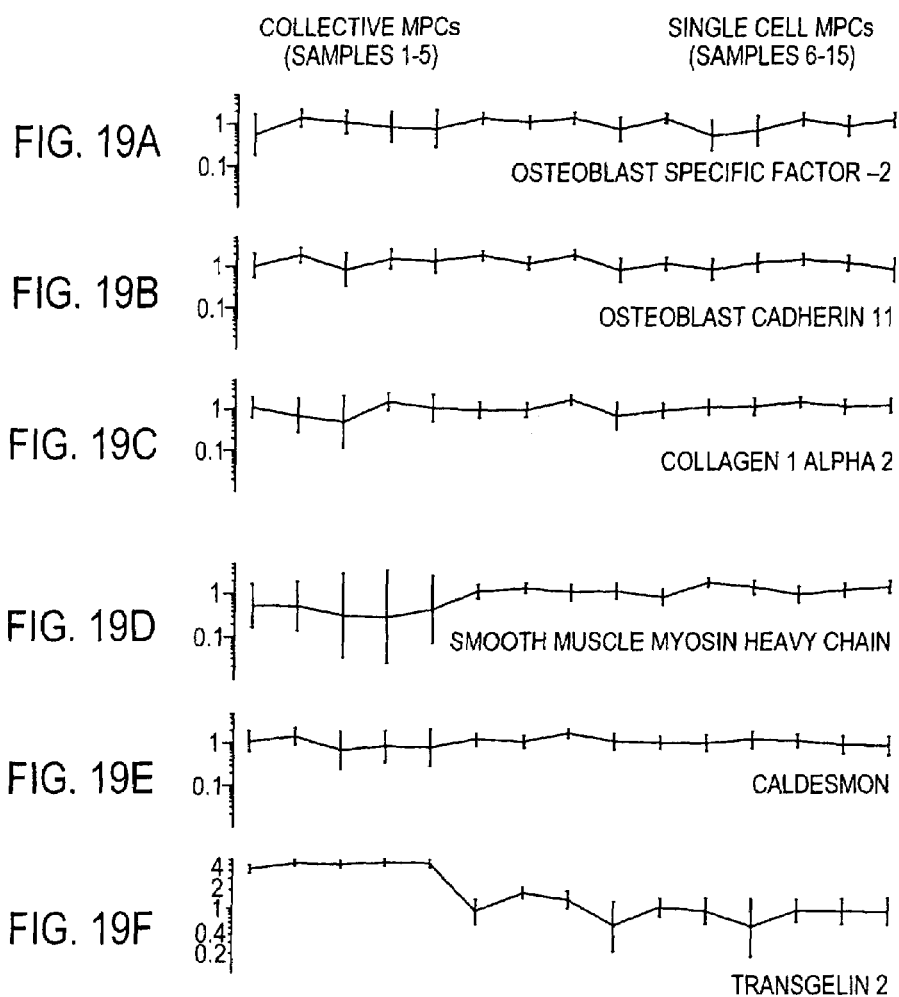
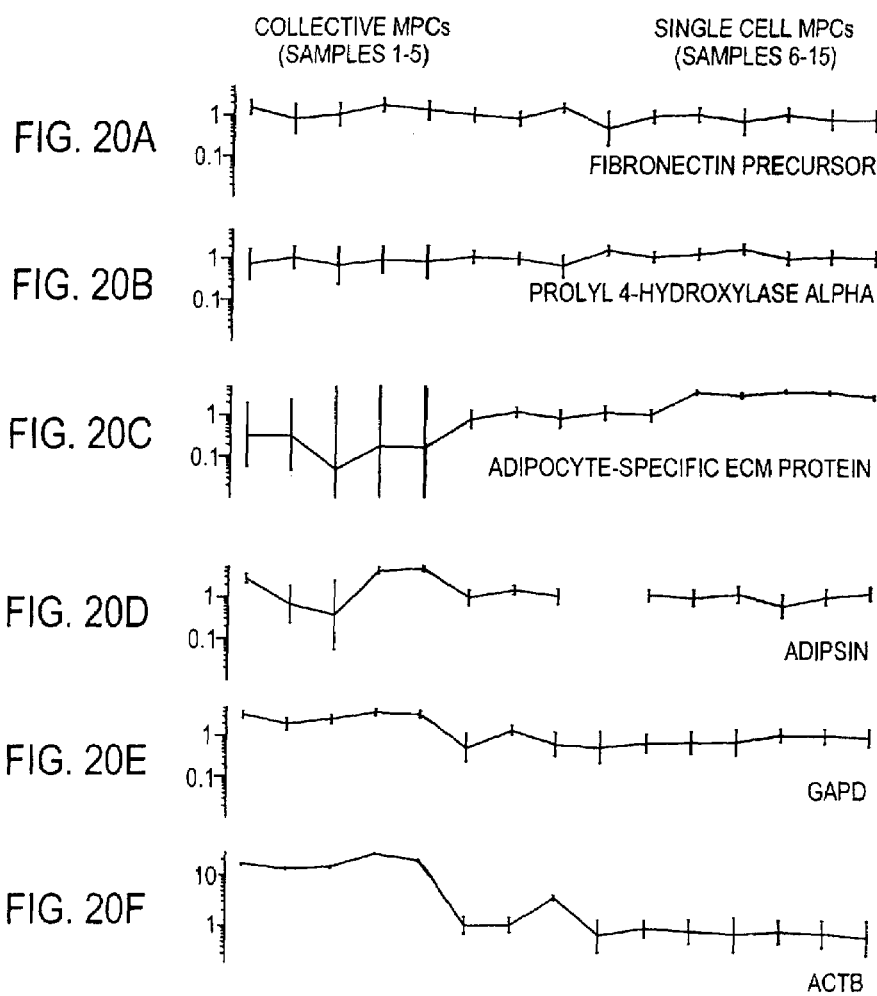
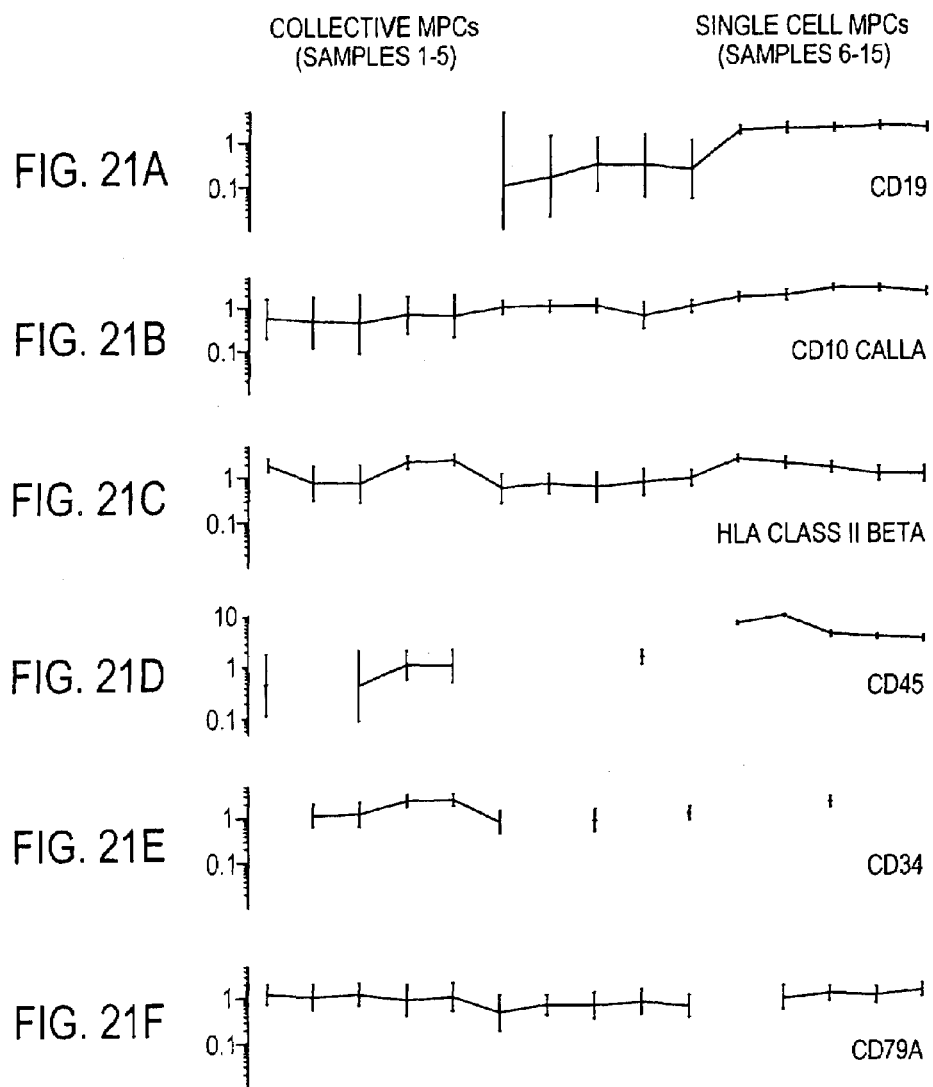


FIG. 18









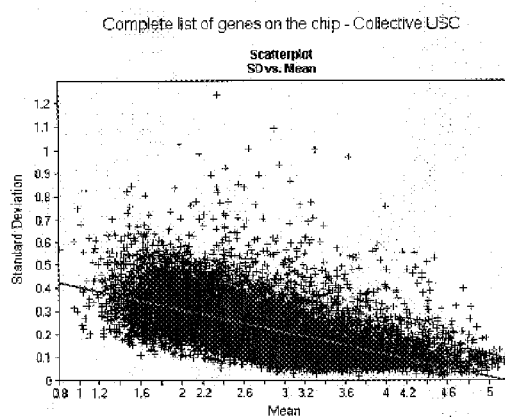


FIG. 22A

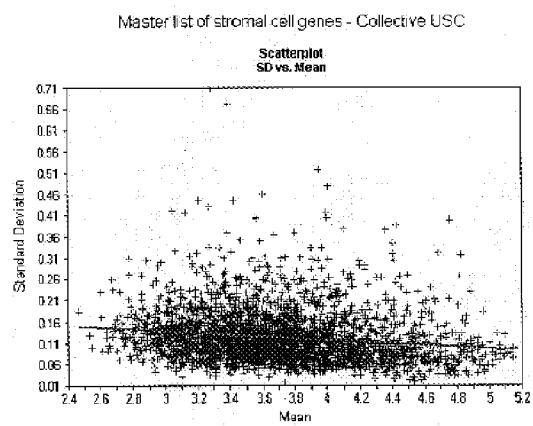


FIG. 22B

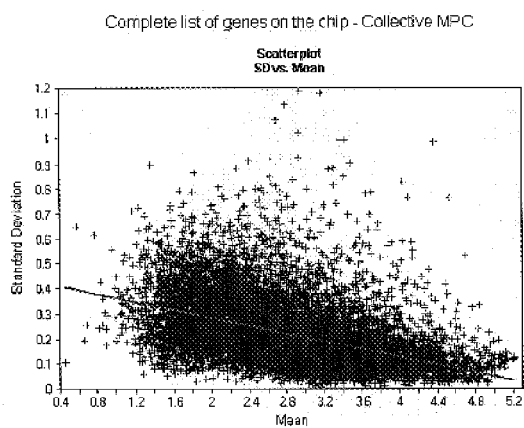


FIG. 22C

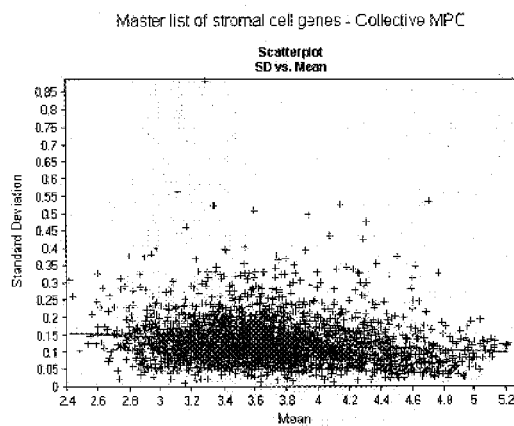


FIG. 22D

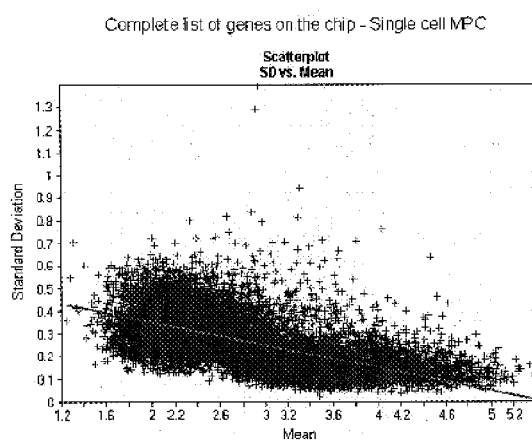


FIG. 22E

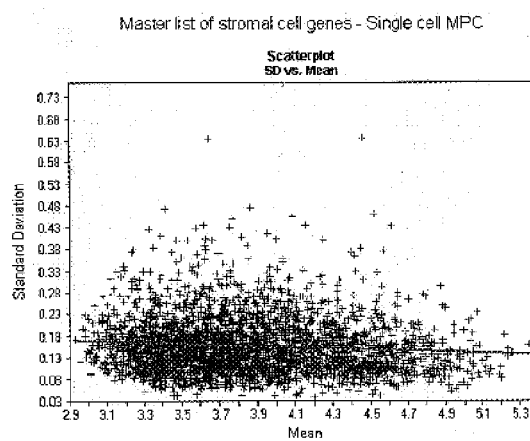


FIG. 22F

# METHOD FOR ENHANCING ENGRAFTMENT OF CELLS USING MESENCHYMAL PROGENITOR CELLS

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Ser. No. 60/486,077, filed Jul. 9, 2003. The present application is also a continuation-in-part of U.S. patent application Ser. No. 10/263,419, filed Oct. 3, 2002, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/327,140, filed Oct. 3, 2001, U.S. Provisional Patent Application Ser. No. 60/334,277, filed Nov. 28, 2001, U.S. Provisional Patent Application Ser. No. 60/352,636, filed Jan. 28, 2002, and U.S. Provisional Patent Application Ser. No. 60/412,450, filed Sep. 20, 2002. U.S. patent application Ser. No. 10/263,419 is also a continuation-in-part of U.S. patent application Ser. No. 09/914,508, filed Nov. 7, 2001 (which is a National Stage Application of International Application Number PCT/US01/16408, filed May 21, 2001, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/277,700, filed Mar. 21, 2001), which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/209,245, filed Jun. 5, 2000. The present application is also a continuation-in-part of U.S. patent application Ser. No. 09/914,508, filed Nov. 7, 2001. Each of the foregoing applications are incorporated herein by reference in their entirety, including all nucleic acid sequences, amino acid sequences, figures, tables, and claims.

## GRANT INFORMATION

The subject matter of this application has been supported by a research grant from the National Heart Lung Blood Institute (NHLBI) and the National Institutes of Health (NIH) under grant number HL59683. Accordingly, the government has certain rights in this invention.

## FIELD OF THE INVENTION

The present invention generally relates to pluri-differentiated mesenchymal progenitor cells and therapeutic uses for the same. More specifically, the isolated mesenchymal progenitor cells are isolated from hematopoietic cells and macrophages in Dexter-type cultures cells.

## BACKGROUND OF THE INVENTION

Bone marrow, the site of blood cell production and home to various leukemia and lymphoma cells, comprises a complex cellular population including hematopoietic progenitor or stem cells and the stromal cells that support them. Hematopoietic stem cells have the capacity for self-regeneration and for generating all blood cell lineages while stromal stem cells have the capacity for self-renewal and for producing the hematopoietic microenvironment.

Two bone-marrow culture systems introduced in the mid-1970's have evolved as favored media for the in vitro analysis of mesengensis and hematopoiesis. The Friedenstein culture system was introduced in 1976 as a media for the analysis and study of mesengensis (Friedenstein, et al, in *Exp Hematol* 4,267-74 (1976)). It is necessary to first isolate rare pluripotent mesenchymal stem cells from other cells in the bone marrow. In the Friedenstein culture system, isolating the non-hematopoietic cells is achieved by utilizing their tendency to adhere to plastic. Once isolated, a monolayer of homoge-

neous, undifferentiated stromal cells is then grown in the culture medium, in the absence of hematopoietic cells. The stromal cells from this system have the potential to differentiate into discrete mesenchymal tissues, namely bone, cartilage, adipose tissue and muscle depending on specific growth supplements. These MSCs have been the target of extensive investigation including exploration of their potential clinical utility in repair or replacement of genetically damaged mesenchymal tissues.

In 1977, Dexter, et al. developed another bone marrow culture system for the study of hematopoiesis (Dexter et al. *J Cell Physiol* 91, 335-44 (1977)). The Dexter culture does not require isolation of the mesenchymal cells before culturing. Thus, the monolayer of stromal cells is grown in the presence of hematopoietic cells. Greenberger later modified the Dexter system by the addition of hydrocortisone to the culture medium, making it more reproducible (Greenberger, *Nature* 275, 752-4 (1978)).

Based on the Dexter system's ability to support sustained growth and preservation of hematopoietic progenitor cells, it has become the standard in vitro model for the study of hematopoiesis. Although the Dexter-type stromal cells and the MSCs in Friedenstein-type cultures express similar cytokine/growth factor profiles, the Dexter cultures have been found to be more efficient at maintaining preservation of hematopoietic progenitor cells. Over the last 23 years, questions have remained as to whether the cells from the Dexter cultures retained the potential to differentiate, like the MSCs in the Friedenstein culture, or whether they have differentiated into another and discrete phenotype due to their interaction with the hematopoietic cells (Prockop, *Science* v276 n5309, p71 (4) (April 1997)). It has been widely believed that the stromal cells of the Dexter cultures are a heterogeneous mixture of adipocytes, osteoblasts, fibroblasts, muscle cells, and vascular endothelial cells.

The in vitro analysis and study of hematopoiesis in Friedenstein and Dexter culture systems has been of great importance in both veterinary and human medicine. A number of diseases and immune disorders, as well as malignancies, appear to be related to disruptions within the hematopoietic system.

Allogeneic bone marrow transplantation is the preferred treatment for a variety of malignant and genetic diseases of the blood and blood-forming cells. The success rate of allogeneic bone marrow transplantation is, in large part, dependent on the ability to closely match the major histocompatibility complex of the donor cells with that of the recipient cells to minimize the antigenic differences between the donor and the recipient, thereby reducing the frequency of host-versus-graft responses and graft-versus-host disease (GvHD). Unfortunately, only about 20% of all potential candidates for bone marrow transplantation have a suitable family member match.

Bone marrow transplantation can be offered to those patients who lack an appropriate sibling donor by using bone marrow from antigenically matched, genetically unrelated donors (identified through a national registry), or by using bone marrow from a genetically related sibling or parent whose transplantation antigens differ by one to three of six human leukocyte antigens from those of the patient. Unfortunately, the likelihood of fatal GvHD and/or graft rejection increases from 20% for matched sibling donors to 50% in the cases of matched, unrelated donors and un-matched donors from the patient's family.

The potential benefits of bone marrow transplantation have stimulated research on the cause and prevention of GvHD. The removal of T cells from the bone marrow obtained from

matched unrelated or unmatched sibling donors results in a decreased incidence of graft versus host reactions, but an increased incidence of rejection of the allogeneic bone marrow graft by the patient.

Current therapy for GvHD is imperfect, and the disease can be disfiguring and/or lethal. Thus, risk of GvHD restricts the use of bone marrow transplantation to patients with otherwise fatal diseases, such as severe immunodeficiency disorders, severe aplastic anemia, and malignancies.

The potential to enhance engraftment of bone marrow or stem cells from antigenically mismatched donors to patients without graft rejection or GvHD would greatly extend the availability of bone marrow transplantation to those patients without an antigenically matched sibling donor.

Thus, it would be useful to develop methods of improving and/or enhance bone marrow transplantation by enhancing the engraftment of bone marrow or hematopoietic progenitor cells and/or decreasing the occurrence of graft rejection or GvHD in allogeneic transplants.

Studies of hematopoiesis and mesengensis and the urgent need for improved methods of treatment in the field of bone marrow transplants have led to the isolation of MSCs from bone marrow stroma. These MSCs are the same pluri-potential cells that result from expansion in Friedenstein type cultures. Several patents describe the isolation and therapeutic uses of these MSCs.

U.S. Pat. No. 5,486,359, to Caplan, et al., discloses isolated human MSCs, and a method for their isolation, purification, and culturing. Caplan, et al. also describes methods for characterizing and using the purified mesenchymal stem cells for research, diagnostic, and therapeutic purposes. The invention in '359, to Caplan, et al., describes pluri-potential cells that remain pluri-potential, even after cultural expansion. Caplan, et al. also teaches that it is necessary to first isolate the pluri-potent MSCs from other cells in the bone marrow and then, in some applications, uses culture medium to expand the population of the isolated MSCs. The Caplan et al. patent fails to disclose the use of Dexter-type cultures, pluri-differentiated mesenchymal progenitor cells, or the isolation of cells from Dexter-type cultures.

U.S. Pat. No. 5,733,542, to Haynesworth, et al., discloses methods and preparations for enhancing bone marrow engraftment in an individual by administering culturally expanded MSC preparations and a bone marrow graft. U.S. Pat. No. 6,010,696, to Caplan, et al., discloses methods and preparations for enhancing hematopoietic progenitor cell engraftment in an individual by administering culturally expanded MSC preparations and hematopoietic progenitor cells. The cells utilized in the Haynesworth, et al. patent and the '696 patent to Caplan, et al. are the pluri-potential cells described in U.S. Pat. No. 5,486,359. Neither patent discloses the use of Dexter-type cultures, pluri-differentiated mesenchymal progenitor cells, or the isolation of cells from Dexter-type cultures.

Mesenchymal stem cells that are isolated from bone marrow are further described by Prockop, in *Science* v276 n5309, p71 (4) (1997) and Pittenger, et al. in *Science* v284 i5411, p143 (1). These articles also describe pluri-potential but undifferentiated MSCs and fail to teach or disclose a pluri-differentiated mesenchymal cell or the isolation of mesenchymal cells from Dexter-type cultures.

While the cells disclosed in the prior art may provide some benefit, the isolated MSCs in the prior art have not solved the problems associated with engraftment of hematopoietic progenitor cells or bone marrow. Consequently, there exists a need in the art for methods of improving engraftment of hematopoietic progenitor cells and bone marrow in mammals

in need of such treatment. There also exists a need in the art for treating and preventing the occurrence of GvHD in mammals that receive allogeneic bone marrow transplants.

#### SUMMARY OF THE INVENTION

According to the present invention there is provided isolated pluri-differentiated mesenchymal progenitor cells, a method of isolation, diagnostic uses, and therapeutic uses relating to enhancing the engraftment of human bone marrow or hematopoietic progenitor cells and treating GvHD.

The present invention provides an isolated mesenchymal progenitor cell that is pluri-differentiated.

Accordingly, the present invention also provides a method for purifying pluri-differentiated mesenchymal progenitor cells including the steps of: providing a cell culture preparation by the Dexter method, treating the cells to obtain a cell suspension, removing macrophages, fractionating the cells, and collecting the fraction of pluri-differentiated mesenchymal progenitor cells.

The present invention also provides a method for enhancing bone marrow engraftment in a mammal in need thereof which includes administering to the mammal (i) isolated pluri-differentiated mesenchymal progenitor cells and (ii) a bone marrow graft, wherein the pluri-differentiated mesenchymal progenitor cells are administered in an amount effective to promote engraftment of the bone marrow in the mammal.

The present invention provides a method for enhancing engraftment of hematopoietic progenitor cells in a mammal in need thereof which includes the step of administering to the mammal (i) isolated pluri-differentiated mesenchymal progenitor cells and (ii) hematopoietic progenitor cells, wherein the pluri-differentiated mesenchymal progenitor cells are administered in an amount effective to promote engraftment of the hematopoietic progenitor cells in the mammal.

Another embodiment of the present invention provides a method for treating graft-versus-host disease (GvHD) in a mammal about to undergo bone marrow or organ transplantation or suffering from GvHD caused by bone marrow or organ transplantation, by administering to the mammal an effective amount of isolated pluri-differentiated mesenchymal progenitor cells.

Yet another embodiment of the present invention provides a method for diagnosing a disease state by: a) establishing gene expression patterns of normal state bone marrow derived isolated pluri-differentiated mesenchymal progenitor cells; b) establishing gene expression patterns of various leukemic state bone marrow derived isolated pluri-differentiated mesenchymal progenitor cells; c) identifying gene sets that are unique to a given state; and d) comparing a profile of bone marrow derived isolated mesenchymal progenitor cell of unknown state to the gene sets.

Additionally, the present invention provides a method for identifying therapeutic targets for treatment of hematopoietic function by: a) determining the median gene expression profile of bone marrow isolated pluri-differentiated mesenchymal progenitor cells associated with each disease state of interest; b) identifying gene groups that are up-regulated, down regulated, and common to each disease state; and c) identifying gene sets that are unique to a given state.

The present invention also includes therapeutic compositions including isolated pluri-differentiated mesenchymal progenitor cells and a pharmaceutically acceptable carrier, wherein the pluri-differentiated mesenchymal progenitor cells are present in an amount effective to enhance bone marrow engraftment in a mammal in need thereof; enhance



hematopoietic progenitor cell engraftment in a mammal in need thereof; or treat GvHD in a mammal about to undergo bone marrow or organ transplantation or suffering from GvHD caused by bone marrow or organ transplantation.

#### BRIEF DESCRIPTION OF THE FIGURES

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Other advantages of the present invention can be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings. The following is a brief description of the drawings which are presented only for the purposes of further illustrating the invention and not for the purposes of limiting same. Referring to the drawing figures, like reference numerals designate identical or corresponding elements throughout the several figures.

FIG. 1 is a photograph showing the phase contrast photomicrograph view of a Dexter-type stromal cell monolayer reflecting on cellular complexity.

FIG. 2 is a photograph showing the percoll gradient centrifugation technique of the present invention that purifies the MPCs (2) in large quantities to greater than 95% purity.

FIG. 3 is a photograph showing the Wright-Giemsa staining of Dexter-type stromal cell cultures depicting three morphologically identifiable cell populations, macrophages (5), hematopoietic cells (3), and the mesenchymal progenitor cells (4) of the present invention.

FIGS. 4A-H show a series of photomicrographs showing the morphologic and phenotypic characteristics of the MPCs of the present invention, as uncovered by staining for representative mesenchymal cell lineage markers. The methods applied are shown in parentheses. (FIG. 4A) Wright-Giemsa (Harleco stain using HMS Series Programmable Slide Stainer, Carl Zeiss, Inc.). (FIG. 4B) Immunostain using anti-CD68 antibody (Immunotech, Clone PG-M1; Vector, Vectastain Elite ABC Kit). (FIG. 4C) Immunostain using anti-CD45 antibody (Dako, Clone PD7/26 & 2B11; ABC Kit). (FIG. 4D) Periodic acid-Schiff (Sigma). (FIG. 4E) Nile Red (Sigma), counterstained with DAPI (Vector). (FIG. 4F) Alkaline phosphatase (Sigma Kit No. 85), counterstained with Nuclear Fast Red (Baker). (FIG. 4G) Immunostain using antibody to fibronectin (Immunotech, Clone 120.5; ABC Kit). (FIG. 4H) Immunostain using anti-muscle actin antibody (Ventana, clone HUC1-1; Ventana system using a section of form alin-fixed, paraffin-embedded cell block, instead of a cytospin). Appropriate positive controls and isotype-matched negative controls were employed to ascertain antibody staining-specificity. All parts of figure as shown, except 4E and 4H, have clearly identifiable built-in cell controls. The morphological features of the cells are listed in row 1 of Table 1.

FIG. 5 is a photograph which shows a transmission electron micrograph of an MPC of the present invention bearing microvilli, irregular nucleus, and pools of glycogen (6) in the ectoplasm ( $\times 4,600$ ).

FIGS. 6A-M are photographs which show Northern blot analysis of bone marrow stromal cell RNAs for expression of genes specific for multiple mesenchymal cell lineages. FIGS. 6A-M represent different gene probes used for hybridization. The following outlines the sources of the gene probes employed and the approximate sizes of the major transcripts observed (shown in parentheses): FIG. 6A) CD68 (Clone ID 3176179, Genome Systems, Inc (GSI); 2-3 kb); FIG. 6B)

Cathepsin B (Clone ID 2806166, GSI; 2-3 kb); FIG. 6C) GAPDH probe (generated using PCR primers from R&D Systems, Inc; ~2 kb) hybridized to same blot as A and B; FIG. 6D) Adipsin (probe generated using PCR primers as described, Ref 20; 0.5-1 kb); FIG. 6E) Osteoblast-specific cadherin-11 (Clone ID 434771, GSI; ~3 kb); FIG. 6F) Chondroitin sulfate proteoglycan 2 (Clone ID 1623237, GSI; >10 kb); FIG. 6G) Collagen type I alpha 1 (Clone ID 782235, GSI; >10 kb); FIG. 6H) Decorin (Clone ID 3820761, GSI; 2-3 kb); FIG. 6I) GAPDH probe hybridized to same blot as D-H; FIG. 6J) Fibronectin (Clone ID 3553729, GSI; >10 kb); FIG. 6K) Caldesmon (Clone ID 1319608, GSI; ~4 kb); FIG. 6L) Transgelin (Clone ID 4049957, GSI; ~1.5 kb); and FIG. 6M) GAPDH probe hybridized to same blot as J-L.

FIG. 7 is a photograph which shows RT-PCR analysis for expression of representative hematopoietic growth factors (G-CSF and SCF) and extracellular matrix receptors (ICAM-1, VCAM-1, and ALCAM) by the MPCs of the present invention.

FIG. 8 is a graph comparing of the ability to support in vitro hematopoiesis by the purified MPCs (heavy fraction represented by gray) of the present invention vs. unfractionated bone marrow stromal cells (represented by black).

FIGS. 9A and 9B are graphs showing flow cytometric evidence of human hematopoietic cell engraftment in a SCID mouse cotransplanted with the MPCs of the present invention. FIG. 9A shows CD45+/CD34+ progenitors in the marrow. FIG. 9B shows CD45/CD34—mature hematopoietic cells circulating in the blood.

FIGS. 10A-H are photographs which show engraftment of human hematopoietic cells in a SCID mouse cotransplanted with the purified marrow MPCs of the present invention. FIG. 10A shows a serial section of a mouse spleen stained with H & E. FIG. 10B shows a serial section of a mouse spleen stained with immunoperoxidase stain for CD45. FIG. 10C shows bone marrow stained for CD45. FIG. 10D shows a serial section of the mouse liver stained with H&E depicting involvement of periportal areas. FIG. 10E shows a serial section of the mouse stomach stained with H&E showing transmural infiltration. FIG. 10F shows a serial section of the mouse lung stained with H&E showing involvement of peribronchial area. FIG. 10G shows a serial section of the mouse pancreas stained with H&E. FIG. 10H shows a serial section of the mouse paravertebral ganglia stained with H&E.

FIG. 11A-C is a photomicrograph of a serial section of the spleen of a normal BALB/C mouse showing white pulp populated by darkly staining lymphocytes (H&E). FIG. 11B is a photomicrograph of the spleen of a SCID mouse showing white pulp largely consisting of lightly staining stromal framework (H&E). FIG. 11C is a photomicrograph of the spleen of a SCID mouse cotransplanted with human bone marrow MNC and the purified bone marrow MPCs of the present invention showing homing (engraftment) of human B cells to white pulp.

FIGS. 12A-C are photographs which show Southern blotting data. FIG. 12A shows that hybridization of sample DNA using a DNA probe specific for human chromosome 17 alpha satellite DNA (p17H8) results in a 2.7 Kb band (7) (arrow; autoradiogram exposed for only 45 minutes). FIG. 12B shows EcoRI digest of thymic genomic DNA from SCID mice. FIG. 12C shows EcoRI digest of lymph node genomic DNA from SCID mice.

FIGS. 13A-1, 13A-2, 13B-1, and 13B-2 show graphs comparing the survival rate and engraftment of human hematopoietic cells in SCID mice cotransplanted with the purified bone marrow MPCs of the present invention vs. unpurified bone marrow stromal cells. In the line graphs provided the

line with diamonds represents MPCs and bone marrow mononuclear cells, squares represents bone marrow mononuclear cells only, triangles represents unfractionated bone marrow stromal cells, the Xs represent MPCs only, and the circles represent the control. In the bar graphs, the gray bars represent mice that survived and the black bars represent mice with engraftment.

FIGS. 14A-D are photographs which demonstrate apoptosis by TUNEL assay in organs of SCID mice that died after transplantation. FIG. 14A shows a serial section of the liver of the mouse that survived. FIG. 14B shows a serial section of the liver of the mouse that died. FIG. 14C shows a serial section of the spleen of the mouse that survived. FIG. 14D shows a serial section of the spleen of the mouse that died.

FIG. 15 shows photomicrographs of single-cell MPCs that were isolated by laser capture microdissection (LCM) and subsequently targeted for microarray analysis.

FIG. 16 shows a Venn diagram displaying the stromal-cell gene-list. Stromal cell genes are operationally defined as being active in at least 9 out of 10 single cell MPCs AND 4 out of 5 collective MPC samples AND 7 out of 8 collective USC samples, i.e., 20 of 23 samples tested. This criterion was very stringent and automatically excluded the outliers, independently of filtering for genes with weak expressions on the basis of control strength (referred to as C or CS). The stromal cell gene list of 2755 includes 13 AFFIX microarray-assay positive controls.

FIG. 17 shows a two-dimensional hierarchical clustering of 2755 stromal cell genes based on the expression profiles of 23 samples. The gene tree is displayed on top and the experiment or sample tree is shown on left. Accordingly, each column represents a particular gene on the chip and each row represents a separate stromal cell sample.

FIG. 18 shows composite gene-expression plots of 2755 stromal cell genes comparing collective purified stromal cell samples (cMPC), collective unpurified stromal cell samples (cUSC) and single-cell stromal cell samples (sMPC). Individual samples are represented on X-axis. Normalized intensity of gene expression is shown on Y-axis in log scale.

FIGS. 19A-19F show gene-expression plots of diverse mesenchymal lineage-associated genes and housekeeping genes by collective MPCs and single-cell MPCs. Individual samples are represented on X-axis. Signal intensity of a transcript in log scale normalized across samples is shown on Y-axis. Note the differing log scales, particularly the wide range of log scale for ACTB. Representative lineage markers are shown as follows. Osteoblast markers: osteoblast-specific factor 2 (probe ID 1451\_s-at), osteoblast cadherin 11 (ID 2087\_s-at) and collagen 1 alpha 2 (ID 32306\_g-at). Muscle markers: caldesmon (ID 41738\_at), transgelin-2 (ID 36678\_at) and smooth muscle myosin heavy chain (ID 32838\_at). Fibroblast markers: fibronectin (ID 31719\_at) and prolyl 4-hydroxylase (ID 37037\_at). Adipocyte markers: adipisin (ID 40282\_at) and adipocyte-specific ECM protein (ID 39673\_i-at). Housekeeping genes: GAPD (ID 35905\_s-at) and ACTB (ID 32318\_s-at). Samples 1-5, respectively, represent MPC A, MPC B R2, MPC C R2, MPC D R1, MPC D R2. Samples 6-15, respectively, represent SCA1, SCA2, SCA3, SCB1, SCB3, SCC1, SCC3, SCD1, SCD2, SCD3.

FIGS. 20A-20F shows gene-expression plots of representative precursor B-lymphocyte-associated genes by collective MPCs and single-cell MPCs. Individual samples are represented on X-axis. Signal intensity of a transcript in log scale normalized across samples is shown on Y-axis. Note that the CD markers that are traditionally associated with hematopoietic cells, CD45 (probe ID 40518\_at), CD19 (ID 1116\_at)

and CD34 (ID 538\_at), are expressed by sMPCs. CD45, when present, is more abundantly detected in single MPCs than in collective MPCs, and is particularly noticeable by wide range of log scale for CD45. The other pre-B cell associated markers that are expressed by sMPCs are CD10 (ID 1389\_at), HLA-Dr (ID 33261\_at) and CD79A (ID 34391\_at). Samples 1-5, respectively, represent MPC A, MPC B R2, MPC C R2, MPC D R1, MPC D R2. Samples 6-15, respectively, represent SCA1, SCA2, SCA3, SCB1, SCB3, SCC1, SCC3, SCD1, SCD2, SCD3.

FIGS. 21A-21F show gene-expression plots of representative precursor B-lymphocyte-associated genes by collective MPCs and single-cell MPCs. Individual samples are represented on X-axis. Signal intensity of a transcript in log scale (normalized across 15 samples) is shown on Y-axis. The CD markers that are traditionally associated with hematopoietic cells, CD45 (probe ID 40518\_at), CD19 (ID 1116\_at) and CD34 (ID 538\_at), are expressed by sMPCs. CD45, when present, is more abundantly detected in single MPCs than in collective MPCs, and is particularly noticeable by wide range of log scale for CD45. The other pre-B cell associated markers that are expressed by sMPCs are CD10 (ID 1389\_at), HLA-Dr (ID 33261\_at) and CD79A (ID 34391\_at). Samples 1-5, respectively, represent MPC A, MPC B R2, MPC C R2, MPC D R1, MPC D R2. Samples 6-15, respectively, represent SCA1, SCA2, SCA3, SCB1, SCB3, SCC1, SCC3, SCD1, SCD2, and SCD3.

FIGS. 22A-22F show scatter plots using log transformed data and showing systematic analysis of transcriptome wide random variation. The methods involved in construction of scatter plots are described in the section entitled, "Second-tier data-analysis/data mining". The results are discussed in the section entitled "Data mining and reproducibility of overall procedures".

#### DETAILED DESCRIPTION OF THE INVENTION

Generally, the present invention provides isolated and purified mesenchymal progenitor cells that are pluri-differentiated. Also provided by the present invention is a therapeutic (pharmaceutical) composition including an effective amount of isolated and purified pluri-differentiated mesenchymal progenitor cells and a pharmaceutically acceptable carrier.

In one aspect, the present invention provides an isolated pluri-differentiated mesenchymal progenitor cell, wherein the cell simultaneously expresses a plurality of genes that are markers for multiple cell lineages, wherein the multiple cell lineages comprise at least four different mesenchymal cell lineages, and wherein each of the markers is specific for a single cell lineage. The terms "mesenchymal progenitor cell", "MPC", and "pluri-differentiated mesenchymal progenitor cell" are used interchangeably herein to refer to the aforementioned cells of the subject invention.

In one embodiment, the MPC is not a cell of a cell line. In another embodiment, the at least four different mesenchymal cell lineages comprise adipocyte, osteoblast, fibroblast, and muscle cell. In another embodiment, the markers are specific for a single cell lineage are selected from the group consisting of Nile Red, Oil Red O, adipisin, alkaline phosphatase, cadherin-11, chondroitin sulfate, collagen type I, decorin, fibronectin, prolyl-4-hydroxylase, actin, caldesmon, and transgelin. In another embodiment, the MPC simultaneously expresses the plurality of genes in the presence of hydrocortisone and horse serum. Preferably, the MPC is not a neoplastic cell, and is chromosomally normal, as determined by Geimsa-trypsin-Wrights (GTW) banding. In one embodiment, the cell is a human cell. The MPC is obtainable directly

from a primary cell culture. Preferably, the primary culture is a Dexter culture. In another embodiment, the MPC is not immortalized.

In another embodiment, the MPC is obtained by providing a cell culture preparation by the Dexter method, treating the cells of the cell culture preparation to obtain a cell suspension, removing macrophages from the cell suspension, fractionating the remaining cells, and collecting the fraction of cells containing the isolated cell. The fractionating step may involve any suitable cell separation technique known in the art, such as fractionation based on density gradient (e.g., Percoll gradient), use of ferromagnetic beads, cytometry, and fluorescence activated cell sorting.

In another aspect, the present invention provides a pharmaceutical composition comprising isolated MPCs and a pharmaceutically acceptable carrier. Preferably, the MPCs are present in an amount effective for treating a disease state in a mammal in need thereof. In one embodiment, the MPC are present in an amount effective to enhance hematopoietic progenitor cell engraftment in a mammal in need thereof. Preferably, the carrier is sterile, such as sterile saline. In another embodiment, the MPC are present in an amount effective to treat graft-versus-host disease (GvHD) in a mammal about to undergo bone marrow or organ transplantation or suffering from GvHD caused by bone marrow or organ transplantation. Optionally, the composition further comprises cells other than MPCs, or tissue, for transplantation. In one embodiment, the tissue comprises bone marrow. In another embodiment, the tissue comprises an organ.

In one embodiment, the MPC of the pharmaceutical composition of the invention are obtained by providing a cell culture prepared by the Dexter method, treating the cells of the cell culture to obtain a cell suspension, removing macrophages from the cell suspension, fractionating the remaining cells, and collecting the fraction containing the pluri-differentiated mesenchymal progenitor cells.

In another aspect, the present invention provides a plurality of isolated MPC (i.e., pluri-differentiated mesenchymal progenitor cells, wherein the plurality of cells are cells that individually simultaneously express a plurality of genes that are markers for multiple cell lineages, wherein the multiple cell lineages comprise at least four different mesenchymal cell lineages, and wherein each of the markers is specific for a single cell lineage). Preferably, the MPCs have been isolated from hematopoietic cells and macrophages to a purity of at least 95%.

In one embodiment, the plurality of isolated MPC are obtained by providing a cell culture preparation by the Dexter method, treating the cells of the cell culture preparation to obtain a cell suspension, removing macrophages from the cell suspension, fractionating the remaining cells, and collecting the fraction of cells containing the plurality of cells.

In another aspect, the present invention provides a method for purifying pluri-differentiated mesenchymal progenitor cells comprising the steps of: (a) providing a cell culture preparation by the Dexter method; (b) treating the cells to obtain a cell suspension; (c) removing macrophages from the cell suspension; (d) fractionating the remaining cells; and (e) collecting the fraction of pluri-differentiated mesenchymal progenitor cells, wherein the pluri-differentiated mesenchymal progenitor cells individually simultaneously express a plurality of genes that are markers for multiple cell lineages, wherein the multiple cell lineages comprise at least four different mesenchymal cell lineages, and wherein each of the markers is specific for a single cell lineage.

In another aspect, the present invention provides a method for enhancing engraftment of cells in a human or non-human

mammal in need thereof, the method comprising administering to the mammal isolated pluri-differentiated mesenchymal progenitor cells of the invention, wherein the isolated pluri-differentiated mesenchymal progenitor cells are administered in an amount effective to promote engraftment of the cells. In one embodiment, the isolated pluri-differentiated mesenchymal progenitor cells are administered by intravenous injection or by injecting directly to the site of intended activity. Optionally, the method further comprises administering the cells for engraftment, wherein the cells are administered before, during, or after the isolated pluri-differentiated mesenchymal progenitor cells are administered. In one embodiment, the cells to be engrafted comprise hematopoietic progenitor cells. In another embodiment, the isolated pluri-differentiated mesenchymal progenitor cells are administered to the mammal in a cell suspension further comprising hematopoietic progenitor cells.

In another aspect, the present invention provides a method for enhancing bone marrow engraftment in a mammal in need thereof, the method comprising administering to the mammal (i) isolated pluri-differentiated mesenchymal progenitor cells of the invention and (ii) a bone marrow graft, wherein the isolated pluri-differentiated mesenchymal progenitor cells are administered in an amount effective to promote engraftment of the bone marrow in the mammal. Advantageously, the isolated pluri-differentiated mesenchymal progenitor cells are capable of increasing the survival of hematopoietic cells transplanted simultaneously or consecutively. In one embodiment, the administering comprises intravenously injecting or directly injecting the isolated pluri-differentiated mesenchymal progenitor cells to the site of intended engraftment.

In another aspect, the present invention provides a diagnostic method for screening isolated pluri-differentiated mesenchymal progenitor cells for abnormalities, comprising isolating RNA from the isolated pluri-differentiated mesenchymal progenitor cells for abnormalities; amplifying the isolated RNA; analyzing the amplified RNA using nucleic acid array (e.g., microarray); determining one or more gene expression patterns; and comparing the determined one or more gene expression patterns to one or more gene expression patterns of normal pluri-differentiated mesenchymal progenitor cells. The diagnostic method may be used to screen for a hematologic disease or other diseases effecting stromal cells, for example. In one embodiment, the abnormalities are phenotypic abnormalities that can be discerned at a single cell level.

Amplification of nucleic acids is typically performed prior to arraying the nucleic acids. Commonly, amplification involves one or more nucleic acid amplifications, e.g., by a PCR, TMA, NASBA or RCA reaction. Optionally, the PCR is an rtPCR that couples reverse transcription and amplification of the expressed RNA samples. The amplification can be either a global amplification or a selective (e.g., target specific) amplification of one or more species in the expressed RNA sample(s). Each expressed RNA sample can be amplified in two or more target specific amplification arrays, and, for example, spatially arrayed in two or more locations on a physical array. Optionally, a plurality of defined sequence probes each of which specifically hybridizes to the products of a different target specific amplification reaction is hybridized to the array. In some embodiments, amplification products are pooled for arraying.

A variety of nucleic acid array formats can be employed in the context of the present invention. In some embodiments, the arrays are solid phase arrays, i.e., the nucleic acids are arrayed on one or more solid phase surface. In some embodiments, the nucleic acids corresponding to expressed RNA

samples are arrayed on a two dimensional solid phase surface. In alternative embodiments, the nucleic acids are arrayed on a plurality of solid phase surfaces, such as beads, spheres, pins, or optical fibers. Solid phase arrays surfaces can include a variety of materials, and in various embodiments of the invention, the array surface is composed, e.g., of glass, coated glass, silicon, porous silicon, nylon, ceramic or plastic. In various embodiments of the invention, expressed RNA samples for analysis are obtained from a variety of biological sources or samples (e.g., bone marrow derived cultures) which have been exposed to or treated with members of a library of compositions (such as cytokines) or agents of potential therapeutic value.

In another aspect, the invention provides a method for reducing graft-versus-host disease (GvHD) in a mammal caused by bone marrow or organ transplantation, the method comprising administering to the mammal an effective amount of isolated pluri-differentiated mesenchymal progenitor cells of the present invention.

In another aspect, the invention provides a method for diagnosing a disease state comprising the steps of: (a) establishing gene expression patterns of normal state bone marrow derived isolated pluri-differentiated mesenchymal progenitor cells; (b) establishing a gene expression pattern for bone marrow derived isolated pluri-differentiated mesenchymal progenitor cells of different leukemic states; (c) identifying gene sets that are unique to a given leukemic state; and (d) comparing a profile of a bone marrow derived isolated mesenchymal progenitor cell of unknown state to the gene sets.

In another aspect, the invention provide a method for diagnosing a disease state in a patient, the method comprising: (a) providing a gene expression profile of a bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell of unknown state from the patient; and (b) comparing the patient gene expression profile to at least one reference gene expression profile to diagnose a disease state in the patient, wherein the reference gene expression profile is a gene expression profile of a bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell in a leukemic state or in a normal state. In one embodiment, the comparing step comprises comparing the patient gene expression profile to a plurality of reference gene expression profiles, wherein each of the reference gene expression profiles is associated with a different leukemic state. Each reference gene expression profile can comprise genes differentially expressed in the leukemic state compared to the normal state.

In one embodiment, the differentially expressed genes comprise at least one class of genes selected from the group consisting of annexins, caspases, cadherins, calmodulins, calmodulin-dependent kinases, cell adhesion molecules, cathepsins, collagens, cytokines, epidermal growth factors, fibroblast growth factors, fibronectins, galectins, growth factors, genes of the IGF system, interleukins, interleukin receptors, integrins, disintegrins, lineage-specific markers, laminins, platelet-derived growth factors, platelet-derived growth factor receptors, interferon-gamma, TNF-alpha, and TGF-beta. In a specific embodiment, the differentially expressed genes comprise TNF-alpha, TGF-beta, and interferon-gamma.

Each reference gene expression profile can comprise expression values of genes differentially expressed in the leukemic state compared to the normal state. In one embodiment, the at least one reference gene expression profile is contained within a database. Preferably, the comparing step is carried out using a computer algorithm. Optionally, the method further comprises (c) selecting the reference gene expression profile most similar to the patient gene expression

profile, to diagnose the patient. Optionally, the method further comprises isolating the bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell of unknown state from the patient.

In one embodiment, the at least one reference gene expression profile comprises a gene expression profile of a bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell in a leukemic state and a gene expression profile of a bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell in a normal state.

Optionally, the method further comprises preparing the patient gene expression profile. The at least one reference gene expression profile can be prepared by cluster analysis, for example.

In another embodiment, the method further comprises: (c) providing a gene expression profile of a bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell from the patient after the patient has undergone a treatment regimen for a leukemic disease state; and (d) comparing the post-treatment patient gene expression profile to the at least one reference gene expression profile, to monitor the patient's response to the treatment regimen.

The leukemic state may be a preleukemic condition, such as myelodysplastic syndrome (MDS). The leukemic can be an over leukemia. The leukemic state can be a lymphoma, for example. In one embodiment, the leukemic state is selected from the group consisting of acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocyte leukemia (CLL), and multiple myeloma (MM).

Optionally, the method may further comprise (c) providing a diagnosis of the disease state to the patient.

In one embodiment, the bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell of unknown state comprises a single cell.

In another embodiment, the bone marrow derived isolated pluri-differentiated mesenchymal progenitor cell of unknown state comprises a plurality of cells.

In one embodiment, the isolated pluri-differentiated mesenchymal progenitor cells have been obtained by providing a cell culture preparation by the Dexter method, treating the cells of the cell culture preparation to obtain a cell suspension, removing macrophages from the cell suspension, fractionating the remaining cells, and collecting the fraction of cells containing the normal state pluri-differentiated mesenchymal progenitor cells.

Typically, isolated pluri-differentiated mesenchymal progenitor cells individually share the characteristic of simultaneously expressing a plurality of genes that are markers for multiple cell lineages, wherein the multiple cell lineages comprise at least four different mesenchymal cell lineages, wherein each of the markers is specific for a single cell lineage, and wherein the cells are not cells of a cell line.

In another aspect, the present invention provides a method for identifying therapeutic targets for treatment of hematopoietic function comprising the steps of: (a) determining the median gene expression profile of isolated pluri-differentiated mesenchymal progenitor cells associated with each disease state of interest; (b) identifying gene groups that are up-regulated, down regulated, and common to each disease state; and (c) identifying gene sets that are unique to a given disease state.

The terms "enhance" or "improve" as used herein are intended to indicate that there is a more beneficial end result. In other words, the product provides a more effective result.

In another aspect, the present invention provides a method of selecting a therapy for a patient, the method comprising: a) providing a subject expression profile of a pluri-differentiated mesenchymal progenitor cell from the patient; b) providing a plurality of reference gene expression profiles, each associated with a therapy, wherein the subject expression profile and each reference profile has a plurality of values, each value representing the expression level of a gene disclosed herein as being expressed in pluri-differentiated mesenchymal progenitor cells; and c) selecting the reference profile most similar to the subject expression profile, to thereby select a therapy for said patient. Optionally, the method further comprises administering the therapy selected in step c) to the patient. In one embodiment, the most similar reference profile is selected by weighting a comparison value for each value of the plurality using a weight value associated with the particular gene. In one embodiment, the subject expression profile has at least twenty values.

The term "pluri-differentiated" as used herein refers to cells that are a single cell type co-expressing genes specific for multiple lineages. The term "pluri-potential" as used herein refers to cells that are undifferentiated and have the potential to be differentiated into discrete mesenchymal tissues.

Dexter-type cultures contain stromal cells that co-express multiple message lineage markers. These pluri-differentiated cells are referred to by the inventor as mesenchymal progenitor cells (MPCs). Disclosed herein is a process for isolating and purifying MPCs from Dexter-type cultures. Purified MPCs provide a sufficiently defined system to permit detailed elucidation of the role of bone marrow in normal and leukemic hematopoiesis.

The present invention also provides various methods for using MPCs to enhance bone marrow transplantation, enhance hematopoietic progenitor cell engraftment, for diagnostic purposes, or for the treatment of GvHD.

The exact cell types in Dexter cultures have now been identified. No evidence was found for the existence of discrete cellular populations, such as adipocytes, osteoblasts, fibroblasts, smooth muscle cells and endothelial cells, notwithstanding the abundance of literature and wide spread belief (See, J. L. Liesveld et al., *Blood* 73, 1794 (1989); A. K. Sullivan, D. Claxton, G. Shematek et al., *Lab Invest* 60, 667 (1989); K. Dorshkind, *Ann Rev Immunol* 8, 126 (1990); S. Perkins, R. A. Fleischman, *Blood* 75, 620 (1990); I. A. Denkers, R. H. Beelen, G. J. Ossenkoppele et al., *Ann Hematol* 64, 210 (1992); P. E. Penn, D. Z. Jiang, R. G. Fei et al., *Blood* 81, 1205 (1993); E. de Wynter et al., *J Cell Sci* 106, 761 (1993); A. Ferrajoli et al., *Stem Cells (Dayt)* 12, 638 (1994); B. R. Clark, A. Keating, *Ann NY Acad Sci* 770, 70 (1995); B. S. Wilkins, D. B. Jones, *Br J Haematol* 90, 757 (1995); S. Gronthos, P. J. Simmons, *J Hematother* 5, 15 (1996); D. Soligo et al., Abstract #3926, *Blood* 94, Supplement 1 (Part 2 of 2), p. 168b, Forty 1<sup>st</sup> Annual Meeting of the American Society of Hematology, New Orleans, La., Dec. 3-7, 1999, M -A. Dorheim et al., *J Cell Physiol* 154, 317 (1993); M. K. Majumdar, M. A. Thiede, J. D. Mosca et al., *J Cell Physiol* 176, 57 (1998); D. J. Prockop, *Science* 276, 71 (1997); R. S. Taichman, S. G. Emerson, *J Exp Med* 179, 1677 (1994); R. S. Taichman, M. J. Reilly, S. G. Emerson, *Blood* 87, 518 (1996); C. M. Verfaillie, in *HEMATOLOGY: Basic Principles and Practice*, R. Hoffman, et al., Eds. (Churchill Livingstone, N.Y., 2000), pp. 140-142.; A. J. Henderson, A. Johnson, K. Dorshkind, *J Immunol* 145, 423 (1990); M. W. Long, J. L. Williams, K. G. Mann, *J Clin Invest* 86, 1387 (1990); P. J. Simmons, S. Gronthos, A. Zannettino et al., *Prog Clin Biol Res* 389, 271 (1994); B. A. Roecklein, B. Torok-Storb, *Blood*

85, 997 (1995); J. Wineman, K. Moore, I. Lemischka et al., *Blood* 87, 4082 (1996); K. A. Kelly, J. M. Gimble, *Endocrinology* 139, 2622 (1998); K. C. Hicok et al., *J Bone Miner Res* 13, 205 (1998); S. R. Park, R. O. Oreffo, J. T. Triffitt, *Bone* 24, 549 (1999); J. E. Dennis et al., *J Bone Miner Res* 14, 700 (1999); and B. Torok-Storb et al., *Ann NY Acad Sci* 872, 164 (1999)). Instead, the inventor determined that there are only three types of cells in Dexter-type cultures, namely, macrophages (~35%), hematopoietic cells (~5%), and a type applicant calls "nonhematopoietic cells" (~60%) (FIG. 3, FIG. 4A, and Table 1).

Bone marrow mesenchymal cells, the nonhematopoietic cells in Dexter type cultures, possess distinctive features that have previously gone unrecognized. There is both direct visual (FIGS. 4A-E and FIG. 5) and molecular biological (FIG. 6) evidence to support the existence of this unique cell type. These findings challenge the prevailing belief that stromal cells derived from Dexter cultures comprise multiple singly-differentiated mesenchymal cell types. Because Dexter cultures represent a primary cell culture system, and not a cell line, these studies indicate that cells in these primary cultures themselves are pluri-differentiated, which has been previously unsuspected. The nonhematopoietic cells of the present invention (MPCs) simultaneously express marker genes specific for multiple mesenchymal cell lineages, including adipocytes, osteoblasts, fibroblasts and smooth muscle cells. As shown in the present disclosure, MPCs can also differentiate into B cells and therefore be useful in affecting the functionality of the immune system.

The MPCs in Dexter type cultures were characterized using a variety of techniques. Cytospins were prepared using aliquots of unfractionated cells for performance of various cytological, cytochemical and immunocytochemical stains. Reactivity patterns of the bone marrow culture cells are outlined in Table 1. FIGS. 4A-E illustrate morphologic and phenotypic characteristics, as uncovered by staining for representative cell lineage markers.

Only rarely have investigators in this field taken the approach of preparing a cell suspension and staining cells on cytospins as was done to characterize the cells of the present invention (Simmons, et al., *Nature* 328, p429-32 (1987)) and no other group has used this method to address the issue of pluri-differentiation by bone marrow stromal cells. Almost all of the published studies in the field, with a rare exception (Simmons, et al., *Nature*, 328, p429-32 (1987)), conducted cytochemical and immunocytochemical staining on layers of stromal cells grown to confluence on coverslips. In this situation, the stromal cultures appear very complex especially in the areas of hematopoietic activity, so-called "cobblestones" with macrophages and hematopoietic cells enmeshed in them. Macrophages and nonhematopoietic cells spread themselves and assume varied shapes when they adhere to and grow on plastic or glass. This spreading further contributes to the perceived heterogeneity and complexity. The complexity precludes a clear morphological visualization of the nonhematopoietic cells and consequently interferes with the determination of what percent of what cell type is positive for any given marker.

In terms of lineage markers, up to 100% of the nonhematopoietic cells or MPCs of the present invention expressed two fat cell markers (Nile Red (FIG. 4E) and Oil Red O); an osteoblast marker (alkaline phosphatase (FIG. 4F)); and two fibroblast markers (fibronectin (FIG. 4G) and prolyl-4-hydroxylase). Greater than 85% of the MPCs were also positive for a muscle marker, actin (FIG. 4H). There was no evidence of expression of endothelial cell differentiation, as judged by immunohistochemical staining for CD34 and CD31.

In addition, the Dexter type stromal cells had not previously been subjected to Periodic Acid-Schiff (PAS) staining, which revealed a strong and uniform positivity by almost 100% of the MPCs studied. This indicates the presence of large stores of glycogen (FIG. 4D). The presence of glycogen (6) was confirmed by electron microscopy (see FIG. 5). In this respect, MPCs are reminiscent of the glycogen-laden reticular cells in the developing bone marrow of human fetuses (observed by L.-T. Chen, L. Weiss, *Blood* 46, 389 (1975)). Glycogen deposition is viewed to be a developmentally regulated process during morphogenesis (H. Ohshima, J. Wartiovaara, I. Thesleff, *Cell Tissue Res.* 297, 271 (1999)).

The MPCs also exhibited cytoplasm compartmentalization into endoplasm and ectoplasm. This morphologic finding sheds light on their internal architecture because of correlation of restricted localization of glycogen and smooth muscle actin to ectoplasm; and the restricted localization of acid phosphatase, alkaline phosphatase, Nile Red, Oil Red O, fibronectin, and prolyl-4-hydrolase to endoplasm.

Additional sets of multiple mesenchymal lineage markers were assessed by Northern blotting of unfractionated cells and purified MPCs to eliminate any observer bias that might be inherent in morphological assessment. FIGS. 6A-M represent different gene probes used for hybridization.

Compared to unfractionated cells, the purified nonhematopoietic cells expressed significantly higher levels of markers representing fat cells (adipsin, FIG. 6D); osteoblasts (osteoblast-specific cadherin-11, chondroitin sulfate, collagen type 1 and decorin, FIGS. 6E-H); fibroblasts (fibronectin, FIG. 6J); and smooth muscle cells (caldesmon and transgelin, FIGS. 6K-L).

Taken together, the morphologic, cytochemical, and immunocytochemical results (FIG. 4A-H and Table 1), and the Northern blotting data (FIGS. 6A-M) indicate that the nonhematopoietic stromal cells of the Dexter cultures co-express markers specific for at least four different mesenchymal cell lineages. Using a variety of techniques, applicant has demonstrated that the MPCs co-express multilineage mesenchymal cell phenotypes, and in this respect the multi- or pluri-differentiated MPCs are distinct from the pluri-potential, but undifferentiated, MSCs of Friedenstein cultures (Prockop, *Science* 276, 71-74 (1997)).

The nonhematopoietic cells of the present invention were purified from the macrophages, the dominant "contaminating" cell type, using a Percoll gradient method developed by applicant. MPCs were purified by the following process: cells from a Dexter-type culture were treated to obtain a cell suspension, the macrophages were removed, and the cells were fractionated using discontinuous Percoll gradient centrifugation (FIG. 2). The isolated MPCs were then collected and washed.

The purity of the nonhematopoietic cells was demonstrated by a near complete absence of two macrophage markers, CD68 and cathepsin B (as shown by Northern blotting data, FIGS. 6A and 6B). As a positive control, bone marrow mononuclear cells rich in myelomonocytic cells abundantly expressed CD68 (lanes 5 & 6, FIG. 6A). The Northern blot results are consistent with a purity estimate of ~95% (vs. 60% in unfractionated samples) based on morphology and immunocytochemical staining for CD68.

MPCs isolated to a purity of approximately 95% can be obtained using methods disclosed herein and Seshi B. et al., 2000, "Human Bone Marrow Stromal Cell: Coexpression of Markers Specific for Multiple Mesenchymal Cell Lineages", *Blood Cells Mol Dis* 26(3):234-246, which incorporated herein by reference in its entirety. The remaining 5% of contaminating cells (macrophages and hematopoietic cells) can

be removed using methods known in the art, such as immunomagnetic separation (IMS) techniques, thereby achieving a purity of greater than 99%. Investigators have successfully used immunomagnetic beads to separate and enrich carcinoma cells from bone marrow and peripheral blood for some time (Naume et al., 1997, "Immunomagnetic Techniques for the Enrichment and Detection of Isolated Breast Carcinoma Cells in Bone Marrow and Peripheral Blood", *J. Hematother.*, 6(2):103-114; Naume et al., 1998, "Increased Sensitivity for Detection of Micrometastases in Bone-Marrow/Peripheral-Blood Stem-Cell Products from Breast-Cancer Patients by Negative Immunomagnetic Separation", *Int. J. Cancer*, 78(5):556-560; Shibata, K. et al., 1998, "Detection of Ras Gene Mutations in Peripheral Blood of Carcinoma Patients Using CD45 Immunomagnetic Separation and Nested Mutant Allele Specification Amplification", *Int. J. Oncol.*, 12(6):1333-1338, each of which are incorporated by reference herein in their entirety).

A purified source of MPCs is desirable for a number of reasons. The relative ease with which large numbers of the MPCs can be purified and their distinctive phenotypic characteristics make them valuable targets for future investigations. Purified MPCs provide a sufficiently defined system to permit detailed elucidation of the role of bone marrow in normal and leukemic hematopoiesis in addition to aiding in bone marrow transplantation.

Another major reason that purified cells are desirable is that Dexter cultures also contain a significant percentage of highly immunogenic macrophages that can cause onset of GvHD after transplantation. The MPCs of the present invention are purified to at least ~95% free of macrophages and hematopoietic cells. Note the increased survival rate in Severe Combined Immunodeficiency Disease (SCID) mice that received purified MPCs versus those that received unfractionated bone marrow stromal cells in FIGS. 13B-1 and 13B-2. This data establishes that stromal cells in combination with engraftment or other similar procedures enhances the effectiveness of the treatment.

The present invention also provides methods of enhancing the engraftment of hematopoietic cells and of enhancing the engraftment of bone marrow. The hematopoietic support capacity of the Dexter-type cultures has been repeatedly demonstrated by a number of investigators. RT-PCR analysis showed that Dexter cultures and Friedenstein cultures expressed a similar pattern of cytokine and growth factor mRNAs; yet, Dexter cultures were found to be more efficient than Friedenstein cultures in achieving preservation of hematopoietic progenitors (Majumdar, et al., *J. Cell. Physiol.* 176, 57-66.). The pluri-differentiated MPC is capable of supporting hematopoiesis, as shown by its ability to express representative hematopoietic growth factors/cytokines, i.e., G-CSF and SCF as well as matrix receptors/hematopoietic cell adhesion molecules, i.e., ICAM-1, VCAM-1 and ALCAM (FIG. 7).

Clarification of the nature of the stromal cells and the ability to purify these cells makes it possible to use them as an adjuvant in bone marrow transplantation following high-dose chemotherapy and radiation therapy. These treatment modalities not only cause damage to the hematopoietic stem cells but also to the supportive stromal cells. However, because the bone marrow microenvironment is destroyed, hematopoietic progenitor cell engraftment is delayed until the stromal environment is restored. As a result, a critical aspect of the current invention is directed to the advantages of transplanting isolated mesenchymal progenitor cells to accelerate the process of stromal reconstruction and ultimately bone marrow engraftment. The stromal cells present in the standard bone

marrow transplant are not sufficient in number and can be supplemented with the cultured MPCs of the present invention.

Yet another embodiment of the current invention provides the use of MPC transplantation to major leukemic conditions, such as acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), chronic myeloid leukemia (CML) and multiple myeloma (MM). This is based on applicant's determination that bone marrow stromal cells in a leukemia patient are functionally and structurally defective, regardless of the damage caused by chemotherapy and radiation therapy. Such defects in bone marrow stromal cells are likely to aid and abet leukemia development. Alternatively, stromal cell defects could be secondarily induced by surrounding leukemia cells, thus contributing to the loss of hematopoietic support function of stromal cells and hematopoietic failure, which is an invariable feature in leukemia. Regardless whether the observed stromal cell defects are primary or secondary to the leukemic process, by reason of their indisputable impact on normal hematopoiesis, these defects remain to be corrected to improve the hematopoietic function.

Stromal cells have never been carefully investigated in terms of genomics in view of the widespread belief that they represent a heterogeneous mixture of cell types. Tissue or cellular heterogeneity presents a major challenge for the application of microarray technology. The purified stromal cells of the present invention represent a single pluridifferentiated MPC which allows for genomic study of the stromal cells and the development of new, more objective diagnostic tools for patients suffering from leukemia conditions.

The present invention provides a comprehensive phenotype of cultured bone marrow stromal cells at single cell level for the first time. These findings pave the road for ultimate identification and investigation of these cells in fresh samples of marrow, normal as well as diseased, in which they occur at a low frequency and are extremely difficult to study at the present time. The development of this phenotype forms the basis for various diagnostic tests including a comprehensive test that can be used to screen for different abnormalities of bone marrow stromal cells in various hematologic diseases and other diseases effecting stromal cells.

Results show that isolated single stromal cells simultaneously express transcripts for osteoblasts, fibroblasts, muscle cells and adipocytes. Furthermore, there is shown that isolated single stromal cells simultaneously express transcripts for epithelial cells and neural/glial cells as well as transcripts for CD45, CD19, CD10, CD79a, and representative proto-oncogenes and transcription factors, typically known to be affiliated with normal and neoplastic hematopoietic cells. These findings are evidence of existence of a progenitor cell that is common to nonhematopoietic mesenchymal cells and hematopoietic cells, particularly B-lymphocytes. "Lineage burst" characterized by simultaneous activation of diverse differentiation pathways within the same cell appears to be the signature profile of a stromal cell, indicating that a "pluripotent" cell is "pluridifferentiated" at the molecular level. That is, prior to a selective and full-fledged lineage differentiation, progenitors express genes associated with multiple lineages to which they might possibly commit, thus providing insight into the molecular basis of cellular plasticity.

Transcriptomic analysis has been undeniably contributing to the molecular definition of new disease categories with demonstrable therapeutic benefit. The present invention contributes to the further definition of the stromal cell by refining its molecular signature. The in vivo identification of the stromal cell and its possible ontogenic variants as they might

occur in different hematological diseases and subsequent targeting of these cells holds the key to ultimately treating some, if not all, of these diseases.

By comprehensively defining the gene expression profile of these cells, the present invention demonstrates the technical applicability of single-cell genomics toward understanding the physiology and pathology of both hematopoietic and nonhematopoietic microenvironments. Classically, the adventitial reticular cells located on the abluminal side of the vascular endothelium within the bone marrow microenvironment were thought to represent the stromal cells or their precursors. As with hematopoietic stem or progenitor cells, the stromal progenitor cells are rare in bone marrow occurring at an estimated frequency of 1 in  $10^5$  nucleated cells. Cultured stromal cells represent the progeny of the stromal cell, and not necessarily the stromal cell itself, for which no in vivo assay exists as yet. The technology of single-cell genomics and the blueprint as described in the present invention allows screening for the abnormalities of bone marrow stromal cells in fresh marrow samples that reflect on the ultimate in vivo context.

The ability to purify culture-expanded MPCs from both normal individuals and patients afflicted with various leukemias also allows testing of the hematopoietic supportive role of MPCs in mice models. These systems provide an in vivo model in which to examine the role of human bone marrow microenvironment in normal and leukemic hematopoiesis.

The SCID mouse model is an ideal system in which to investigate MPC function. Engraftment of human hematopoietic progenitors in SCID mice has required either coadministration of exogenous human cytokines, or cotransplantation of human bone marrow plugs or bone fragments. As disclosed herein MPCs are a convenient, new source for human bone marrow stromal cells for enhancing transplantation that does not require cytokines, bone fragment, or marrow.

Unlike prior methods, the isolated MPCs of the present invention support human hematopoiesis in the SCID mouse model as effectively as whole marrow stroma. The transplantation of human marrow mononuclear cells combined with purified MPCs results in dramatically vigorous engraftment of human cells in spleen, bone marrow, liver, pancreas, lungs, stomach, and paravertebral neuronal ganglia of SCID mice (FIGS. 10A-H and FIGS. 11A-C). By contrast, mice receiving human bone marrow mononuclear cells alone or MPCs alone expectedly showed no detectable evidence of human hematopoietic cell engraftment (FIGS. 13A-1, 13A-2, 13B-1, and FIG. 13B-2).

The present invention also provides for a method of preventing or treating GvHD. The highest mortality rate, FIG. 13B, was observed in mice receiving the unpurified whole marrow stroma and the bone marrow mononuclear cells. The increased mortality observed is related to the presence of highly immunogenic macrophages and consequent GvHD. The mice with the highest survival rate, shown in FIG. 13A, were the mice receiving purified MPCs and bone marrow mononuclear cells.

Notably, there is discrete TUNEL-positive nuclei in the liver of the expired mouse in FIG. 14B and complete absence of staining in the liver of the surviving mouse (see FIG. 14A). While some ill-defined globules of staining are observed in the spleen of the mouse that survived, the nuclear integrity of most of the cells is well preserved suggesting minimal or no apoptosis (FIG. 14C). In contrast, the dead mouse spleen (FIG. 14D) showed extensive TUNEL positivity precluding accurate interpretation. Control mouse liver and spleen showed results similar to those of the mouse that survived.



The above results indicate that purified MPCs can support human hematopoiesis in SCID mice as effectively as whole marrow stroma. Equally important is that the purified MPCs increased the survival rate. The evidence shows that the increased survival is due to a reduction in GvHD.

Allogeneic bone marrow transplantation is the preferred method of treatment for a variety of malignant and genetic diseases of the blood and blood forming cells. However, failure of hematopoietic cell engraftment can occur for a number of reasons. These include, microenvironmental defects as part of the underlying disease itself (e.g., aplastic anemia), and/or stromal cell damage caused by chemoradiotherapy and/or microenvironmental damage as part of GvHD which is a dreaded complication following bone marrow transplantation. In GvHD, donor T cells present in the hematopoietic cell graft destroy host tissues. GvHD can involve multiple organs such as skin, liver, GI system etc. The current treatment modalities for preventing or treating graft failure or GvHD are cumbersome, costly and involve some form of immunosuppression. Stromal cell lesions, either primary to the disease process or secondarily induced by allogeneic bone marrow transplantation, play a prominent role in the success or failure of the hematopoietic cell graft. Cotransplantation of MPC not only enhances hematopoietic cell engraftment but also prolongs the life of graft recipients by minimizing GvHD. Co-transplantation of healthy, culture-expanded MPC is a viable option in these situations. The human bone marrow used in the Dexter-type cultures of the present invention can be obtained from a number of different sources in accordance with the procedures known in the art, including from plugs of femoral head cancerous bone pieces or from aspirated marrow. The cells used in the Dexter culture can be autologous, from the tissue donor, or from other individuals.

Modes of administration of MPCs include, but are not limited to, systemic intravenous injection and injection directly to the intended site of activity. The MPCs can be administered by any convenient route, for example by infusion or bolus injection, and can be administered together with other biologically active agents. Administration is preferably systemic.

The methods of the present invention can be altered, particularly by (1) increasing or decreasing the time interval between administering MPCs and implanting the tissue, cells, or implanting the organs; (2) increasing or decreasing the amount of MPCs administered; (3) varying the number of MPC administrations; (4) varying the method of delivery of the MPCs; and/or (5) varying the source of MPCs.

The MPC preparations are used in an amount effective to promote engraftment of hematopoietic progenitor cells or bone marrow cells; or for the treatment or prevention of GvHD in the recipient. The pharmaceutically effective amount for the purposes herein is thus determined by such considerations as are known in the art. In general, such amounts are typically at least  $1 \times 10^4$  MPCs per kg of body weight and most generally need not be more than  $7 \times 10^5$  MPCs/kg.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of MPCs and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to McCoy's medium, saline, buffered saline, dextrose, water, and combinations thereof. In one embodiment, the pharmaceutically acceptable carrier is pharmaceutical grade water or saline. The formulation should suit the method of administration as is known by those of skill in the art. The composi-

tion may be liquid (such as an injectable cell suspension), semi-solid, or solid (such as a tissue scaffold).

In one embodiment, the MPC preparation or composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition can also include a local anesthetic to ameliorate any pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a cryopreserved concentrate in a hermetically sealed container such as an ampoule indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

The pharmaceutical compositions of the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Sciences* (Martin E W, Easton Pa., Mack Publishing Company, 19<sup>th</sup> ed., 1995)) describes formulations which can be used in connection with the subject invention. Formulations suitable for parenteral administration include, for example, aqueous sterile injection solutions, which may contain antioxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions which may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. It should be understood that in addition to the ingredients particularly mentioned above, the compositions of the subject invention can include other agents conventional in the art having regard to the type of formulation in question. For example, in addition to isolated mesenchymal progenitor cells of the subject invention (MPCs) and a pharmaceutically acceptable carrier, the composition may further comprise cells other than isolated mesenchymal progenitor cells, or tissue, for co-transplantation. As used herein, the terms "transplanting", "implanting", "administering", and grammatical variations thereof are used herein interchangeably to refer to the delivery of the particular agent (e.g., cells or composition) systemically or to a target site within the subject.

The MPCs of the present invention may be administered to a subject, such as a human or non-human mammal (e.g., the mouse model of the subject invention, in conjunction with other therapeutic agents, such as anti-cancer agents, cytotoxic agents, and/or chemotherapeutic agents. The MPCs of the present invention may be administered to the subject in conjunction with, or in the absence of, immunosuppressive treatment.

As used herein, the term "anti-cancer agent" refers to a substance or treatment that inhibits the function of cancer cells, inhibits their formation, and/or causes their destruction in vitro or in vivo. Examples include, but are not limited to, cytotoxic agents (e.g., 5-fluorouracil, TAXOL) and anti-signaling agents (e.g., the PI3K inhibitor LY).



As used herein, the term "cytotoxic agent" refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells in vitro and/or in vivo. The term is intended to include radioactive isotopes (e.g.,  $\text{At}^{211}$ ,  $\text{I}^{131}$ ,  $\text{I}^{125}$ ,  $\text{Y}^{90}$ ,  $\text{Re}^{186}$ ,  $\text{Re}^{188}$ ,  $\text{Sm}^{153}$ ,  $\text{Bi}^{212}$ ,  $\text{P}^{32}$ , and radioactive isotopes of Lu), chemotherapeutic agents, toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, and antibodies, including fragments and/or variants thereof.

As used herein, the term "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer, such as, for example, taxanes, e.g., paclitaxel (TAXOL, BRISTOL-MYERS SQUIBB Oncology, Princeton, N.J.) and doxorubicin (TAXOTERE, Rhone-Poulenc Rorer, Antony, France), chlorambucil, vincristine, vinblastine, anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston), and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin, etc.

The terms "comprising", "consisting of", and "consisting essentially of" are defined according to their standard meaning. The terms may be substituted for one another throughout the instant application in order to attach the specific meaning associated with each term. The phrases "isolated" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany the material as it is found in its native state. Thus, isolated pluripotent mesenchymal progenitor cells of the present invention (MPCs) preferably do not contain materials normally associated with the cells in their in situ environment, such as hematopoietic cells and macrophages. In one embodiment, the MPCs are at least 95% pure. In another embodiment, the MPCs are at least 99% pure.

As used herein, the terms "treat" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change or disorder, such as the development or spread of cancer. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those subjects (e.g., human or veterinary patients) in need of treatment include those already with the condition or disorder as well as those prone to have the condition or disorder or those in which the condition or disorder is to be prevented.

As used herein, the term "(therapeutically) effective amount" refers to an amount of an agent (e.g., a cell or composition) effective to treat a disease or disorder in a human or non-human mammal.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "an isolated pluripotent mesenchymal progenitor cells" or "an MPC" includes more than one such cell.

The present invention paves the way for applications of mesenchymal progenitor cells in the field of transplantation with respect to hematopoietic support, immunoregulation, and graft facilitation. MPCs can be used as a supporting cell type in bone marrow transplantation, particularly in diseases where defects in the hematopoietic stromal microenviron-

ment are believed to prevail, such as aplastic anemia, myelofibrosis, and bone marrow failure following high dose chemotherapy and radiation therapy.

Another aspect of the invention provides a method for diagnosing various disease states in mammals by identifying new diagnostic markers, specifically the classification and diagnosis of leukemia. Prior to the present invention, stromal cells were not carefully investigated in terms of genomics because of the widespread belief that they represent a heterogeneous mixture of cell types and cellular heterogeneity presents significant challenges for the application of genetic analysis such as microarray technology. The isolated MPCs of the present invention represent a single cell type and allow for genomic study of the stromal cells.

Using the methods of the present invention, it has been determined that bone marrow stromal cells in leukemia patients are functionally and structurally defective regardless of the damage caused by chemotherapy and radiation therapy. Given the almost 25 year history and intense interest in bone marrow stromal cell cultures, previous documentation of stromal cell abnormalities has been disappointingly low (Martinez & Martinez, *Exp. Hematol* 11:522-26 (1983); Budak-Alpdogan, et al., *Am. J. Hematol*, 62:212-20 (1999); Nagao, et al., *Blood*, 61:589-92 (1983); Peled, et al., *Exp. Hematol* 24:728-37 (1996); Bhatia, et al., *Blood* 85:3636-45 (1995); Agarwal, et al., *Blood* 85:1306-12 (1995); Diana, et al., *Blood* 96:357a(2000)). By identifying gene sets that are unique to a given state, these differences in the stromal cells can be utilized for diagnostic purposes.

In one embodiment of the invention, isolated MPCs from a patient are assayed for expression of a large number of genes. The gene expression profile is projected into a profile of gene set expression values according to the definition of gene sets. A reference database containing a number of reference projected profiles is also created from the isolated MPCs of patients with known states, such as normal and various leukemic disease states. The projected profile is then compared with the reference database containing the reference projected profiles. If the projected profile of the patient matches best with the profile of a particular disease state in the database, the patient is diagnosed as having such disease state. Various computer systems and software (see Example 5) can be utilized for implementing the analytical methods of this invention and are apparent to one of skill in the art. Some of these software programs include Cluster & TreeView (Stanford), GeneCluster (MIT/Whitehead Institute), Array Explorer (SpotFire Inc.) and GENESPRING (Silicon Genetics Inc.) (for computer systems and software, see also U.S. Pat. No. 6,203,987, which is incorporated herein by reference in its entirety).

The methods of the present invention can also be useful for monitoring the progression of diseases and the effectiveness of treatments. For example, by comparing the projected profile prior to treatment with the profile after treatment.

One aspect of the present invention provides methods for therapeutic and drug discovery utilizing bone marrow derived isolated mesenchymal progenitor cells. The present invention can be utilized to identify stromal cell genes that can be therapeutic targets for improvement of normal hematopoietic function, which is constantly compromised, in leukemic patients. In one embodiment, gene sets are defined using cluster analysis. The genes within a gene set are indicated as potentially co-regulated under the conditions of interest. Co-regulated genes are further explored as potentially being involved in a regulatory pathway. Identification of genes involved in a regulatory pathway provides useful information for designing and screening new drugs.

Some embodiments of the present invention employ gene set definition and projection to identify drug action pathways. In one embodiment, the expression changes of a large number of genes in response to the application of a drug are measured. The expression change profile is projected into a gene set expression change profile. In some cases, each of the gene sets represents one particular pathway with a defined biological purpose. By examining the change of gene sets, the action pathway can be deciphered. In some other cases, the expression change profile is compared with a database of projected profiles obtained by perturbing many different pathways. If the projected profile is similar to a projected profile derived from a known perturbation, the action pathway of the drug is indicated as similar to the known perturbation. Identification of drug action pathways is useful for drug discovery. See, Stoughton and Friend, *Methods for Identifying Pathways of Drug Action*, U.S. patent application Ser. No. 09/074,983; U.S. Patent Publication 2001/0018182, filed Feb. 14, 2001; U.S. Patent Publication 2002/0128781, filed Jan. 28, 2002; U.S. Patent Publication 2003/0093227, filed Nov. 4, 2002; U.S. Pat. No. 6,468,476, filed Oct. 27, 1999; U.S. Pat. No. 6,351,712, filed Dec. 28, 1998; and U.S. Pat. No. 6,218,122, filed Jun. 16, 1999, which are incorporated herein by reference in their entirety.

The present invention provides a genomics strategy method for identifying genes differentially expressed in MPCs. The method begins with the preparation of total RNA from MPC samples, which leads to the generation of cDNA. From the cDNA, ds DNA can be prepared for in vitro transcription into cRNA. The cRNA is then fragmented for the hybridization of target RNA to a microarray of known genes (Affymetrix genechip containing DNA from ~12,000 known human genes, e.g., U95A oligonucleotide microarray). Finally, analysis of differentially expressed genes is accomplished using appropriate software (GENESPRING) to discern the patterns of gene expression or genomic signatures by a given MPC type (e.g., up-regulation or down-regulation).

Up-regulated and down-regulated gene sets for a given disease-associated or cytokine-stimulated MPC are combined. The combination enables those of skill in the art to identify gene sets with minimal number of elements that are unique to a given MPC type with a capability to discriminate one MPC type from another (this can be accomplished by means of a series of Venn diagrams and lists of required genes obtained via GENESPRING). Such gene sets are of immense diagnostic value as they can be routinely used in assays that are simpler than microarray analysis (for example "real-time" quantitative PCR). Such gene sets also provide insights into pathogenesis and targets for design of new drugs. For example, the method allows one to establish transcriptional profiles of MPC genes that are pathologically altered.

Those of skill in the art can use the data and methods contained herein for the following: a) study select gene or sets of genes that are relevant to hematopoietic disease conditions by using relatively inexpensive but low-throughput technologies such as Northern blotting, RNase protection assays and/or PCR intended for gene expression analysis; b) identify newer drug targets and diagnostic markers relevant to specific diseases, such as MM or CML etc depending on the research interests of the individual investigators.

The present invention also provides a large-format 2-D gel electrophoretic system for the reproducible separation of MPC proteins and for preparing 2-D PAGE protein maps for normal bone marrow-derived MPCs (untreated and treated with representative cytokines, e.g., TNF- $\alpha$  and/or IL-4) and for MPCs derived from patients with representative pre-leukemic/premalignant and leukemic/malignant conditions. The

pre-leukemic conditions include myelodysplastic syndromes (MDS) and the leukemic conditions include chronic myeloid leukemia (CML), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and multiple myeloma (MM). The protein samples consist of culture supernatants/secreted proteins, extracellular matrix (ECM) proteins, plasma membrane proteins solubilized using a three-step differential extraction protocol, employing conditions of progressively increasing solubility; and whole cell lysate proteins similarly solubilized using the three-step differential extraction protocol. This subproteome approach not only simplifies the 2-D PAGE electrophoretic protein patterns but also reveals additional proteins, which would otherwise have gone undetected.

The 2-D system described herein utilizes an immobilized pH gradient gel (pH 4-7) in the first dimension and a mini non-denaturing but high-resolution lithium dodecyl sulfate-polyacrylamide gel electrophoresis (LDS-PAGE) in the second dimension. As identified by silver staining, this system has resolved greater than 800 protein spots in a pH interval of 2.5 units (4.25-6.75, the isoelectric pH range for most of plasma membrane proteins to migrate) and a molecular mass range of 10-150 kDa. Equally important, the system is compatible with high sample loads (up to 1.5 to 2.0 mg of total protein in up to 350  $\mu$ l sample volume). All the protein species identifiable by a silver stain that is compatible with subsequent mass spectrometric analysis have been analyzed by a 2-D gel software with respect to isoelectric point, molecular weight and mass abundance. The lectin-binding status of these proteins has also been determined by lectin blotting. Lectin blots and Western blots have subsequently been stained by a gold stain for detection of total proteins on the same PVDF membrane. Although gold-staining of the Western blot is not as sensitive as silver-staining of the gel, gold-staining of the Western blot generates the necessary landmarks for alignment with the silver stained gel, facilitating excision of spots of interest from the gel for identification by MALDI-MS. Representative protein spots were excised from gel and subjected to mass spectrometric profiling (MALDI-MS) and/or sequencing (Nano ESI MS/MS) with subsequent database searching, resulting in a productive identification of ten proteins.

As used herein, the term "expression products" refers to ribonucleic acid (RNA) or polypeptide products transcribed or translated, respectively, from a genome or other genetic element. Commonly, expression products are associated with genes having biological properties. Thus, the term "gene" refers to a nucleic acid sequence associated with a biological properties, e.g., encoding a gene product with physiologic properties. A gene optionally includes sequence information required for expression of the gene (e.g., promoters, enhancers, etc.).

As used herein, the terms "expression" or "gene expression" refer to transcription of a gene into an RNA product, and optionally to translation into one or more polypeptide sequences. The term "transcription" refers to the process of copying a DNA sequence of a gene into an RNA product, generally conducted by a DNA-directed RNA polymerase using DNA as a template.

As used herein, the term "nucleic acid" refers to a polymer of ribonucleic acids or deoxyribonucleic acids, including RNA, mRNA, rRNA, tRNA, small nuclear RNAs, cDNA, DNA, PNA, RNA/DNA copolymers, or analogues thereof. Nucleic acid may be obtained from a cellular extract, genomic or extragenomic DNA, viral RNA or DNA, or artificially/chemically synthesized molecules.

As used herein, the term “RNA” refers to a polymer of ribonucleic acids, including RNA, mRNA, rRNA, tRNA, and small nuclear RNAs, as well as to RNAs that comprise ribonucleotide analogues to natural ribonucleic acid residues, such as 2-O-methylated residues.

As used herein, the term “cDNA” refers to complementary or “copy” DNA. Generally cDNA is synthesized by a DNA polymerase using any type of RNA molecule (e.g., typically mRNA) as a template. Alternatively, the cDNA can be obtained by directed chemical syntheses.

As used herein, the term “amplified product” or “amplified nucleic acid” refers to a nucleic acid generated by any method of nucleic acid amplification.

As used herein, the term “complementary” refers to nucleic acid sequences capable of base-pairing according to the standard Watson-Crick complementary rules, or being capable of hybridizing to a particular nucleic acid segment under relatively stringent conditions. Nucleic acid polymers are optionally complementary across only portions of their entire sequences.

As used herein, the term “hybridization” refers to duplex formation between two or more polynucleotides, e.g., to form a double-stranded nucleic acid. The ability of two regions of complementarity to hybridize and remain together depends of the length and continuity of the complementary regions, and the stringency of hybridization conditions.

As used herein, the term “defined sequence probe” is a nucleic acid probe having a single polynucleotide sequence.

As used herein, the term “synthetic probe” is used to indicate that the probe is produced by one or more synthetic or artificial manipulations, e.g., restriction digestion, amplification, oligonucleotide synthesis, cDNA synthesis, and the like.

As used herein, the term “label” refers to any detectable moiety. A label may be used to distinguish a particular nucleic acid from others that are unlabeled, or labeled differently, or the label may be used to enhance detection.

As used herein, the term “primer” refers to any nucleic acid that is capable of hybridizing at its 3' end to a complementary nucleic acid molecule, and that provides a free 3' hydroxyl terminus which can be extended by a nucleic acid polymerase.

As used herein, the term “template” refers to any nucleic acid polymer that can serve as a sequence that can be copied into a complementary sequence by the action of, for example, a polymerase enzyme.

As used herein, the term “target,” “target sequence,” or “target gene sequence” refers to a specific nucleic acid sequence, the presence, absence or abundance of which is to be determined. In a preferred embodiment of the invention, it is a unique sequence within the mRNA of an expressed gene.

As used herein, the term “gene expression data” refers to one or more sets of data that contain information regarding different aspects of gene expression. The data set optionally includes information regarding: the presence of target-transcripts in cell or cell-derived samples; the relative and absolute abundance levels of target transcripts; the ability of various treatments to induce expression of specific genes; and the ability of various treatments to change expression of specific genes to different levels.

As used herein, the term “quantitating” means to assign a numerical value, e.g., to a hybridization signal. Typically, quantitating involves measuring the intensity of a signal and assigning a corresponding value on a linear or exponential numerical scale.

As used herein, the term “algorithm” refers to a set of rules for describing a biological condition. The rule set may be defined exclusively algebraically but may also include alter-

native or multiple decision points requiring domain-specific knowledge, expert interpretation or other clinical indicators.

As used herein, the term “baseline profile data set” refers to a set of values associated with constituents of a gene expression panel resulting from evaluation of a biological sample (or population of samples) under a desired biological condition that is used for mathematically normative purposes. The desired biological condition may be, for example, the condition of a subject (or population of subjects) before exposure to an agent or in the presence of an untreated disease or in the absence of a disease. Alternatively, or in addition, the desired biological condition may be health of a subject or a population of subjects. Alternatively, or in addition, the desired biological condition may be that associated with a population of subjects selected on the basis of at least one of age group, gender, ethnicity, geographic location, diet, medical disorder, clinical indicator, medication, physical activity, body mass, and environmental exposure.

As used herein, the term “biological condition” of a subject is the condition of the subject in a pertinent realm that is under observation, and such realm may include any aspect of the subject capable of being monitored for change in condition, such as health, disease including cancer; trauma; aging; infection; tissue degeneration; developmental steps; physical fitness; obesity, and mood. As can be seen, a condition in this context may be chronic or acute or simply transient. Moreover, a targeted biological condition may be manifest throughout the organism or population of cells or may be restricted to a specific organ (such as skin, heart, eye or blood), but in either case, the condition may be monitored directly by a sample of the affected population of cells or indirectly by a sample derived elsewhere from the subject. The term “biological condition” includes a “physiological condition, such as a hematologic disease or other disease affecting stromal cells, and leukemic states such as pre-leukemic conditions (e.g., myelodysplastic syndrome (MDS), overt leukemia, lymphoma, acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), chronic lymphocyte leukemia (CLL), and multiple myeloma (MM).

As used herein, the term “calibrated profile data set” is a function of a member of a first profile data set and a corresponding member of a baseline profile data set for a given constituent in a panel.

As used herein, a “clinical indicator” is any physiological datum used alone or in conjunction with other data in evaluating the physiological condition of a collection of cells or of an organism. This term includes pre-clinical indicators.

As used herein, to “derive” or “prepare” a profile data set, such as a gene expression profile, from a sample includes determining a set of values associated with constituents of a gene expression panel either (i) by direct measurement of such constituents in a biological sample or (ii) by measurement of such constituents in a second biological sample that has been exposed to the original sample or to matter derived from the original sample.

As used herein, the term “distinct RNA or protein constituent” in a panel of constituents is a distinct expressed product of a gene, whether RNA or protein. An “expression” product of a gene includes the gene product whether RNA or protein resulting from translation of the messenger RNA.

As used herein, the term “gene expression panel” refers to an experimentally verified set of constituents, each constituent being a distinct expressed product of a gene, whether RNA or protein, wherein constituents of the set are selected so that their measurement provides a measurement of a targeted biological condition.

As used herein, the term "gene expression profile" refers to a set of values associated with constituents of a gene set or gene expression panel resulting from evaluation of a biological sample (or population of samples). For example, a gene expression profile can have a minimum number of values selected from the group consisting of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, and 20, or more. According to the methods of the invention, two or more gene expression profiles can be compared. The most similar reference profile can be selected by weighting a comparison value for each value of the plurality using a weight value associated with the corresponding gene.

As used herein, the term "index" is an arithmetically or mathematically derived numerical characteristic developed for aid in simplifying or disclosing or informing the analysis of more complex quantitative information. A disease or population index may be determined by the application of a specific algorithm to a plurality of subjects or samples with a common biological condition.

A "large number" of data sets based on a common panel of genes is a number of data sets sufficiently large to permit a statistically significant conclusion to be drawn with respect to an instance of a data set based on the same panel.

As used herein, a "normative" condition of a subject to whom a composition is to be administered means the condition of a subject before administration, even if the subject happens to be suffering from a disease.

As used herein, a "panel" of genes means a set of genes (a "gene set") including at least two constituents.

A "sample" from a subject may include a single pluridifferentiated mesenchymal progenitor cell or a plurality of pluridifferentiated mesenchymal progenitor cells taken from the subject, by any means known in the art. The sample may be obtained directly from the subject or from primary culture, such as Dexter culture.

As used herein, the term "signature profile" means an experimentally verified subset of a gene expression profile selected to discriminate a biological condition, agent or physiological mechanism of action.

As used herein, the term "signature panel" refers to a subset of a gene expression panel, the constituents of which are selected to permit discrimination of a biological condition, agent or physiological mechanism of action.

As used herein, the term "therapy" includes all interventions whether biological, chemical, physical, metaphysical, or combination of the foregoing, intended to sustain or alter the monitored biological condition of a subject.

Gene expression panels may be used for measurement of therapeutic efficacy of natural or synthetic compositions or stimuli that may be formulated individually or in combinations or mixtures for a range of targeted physiological conditions; prediction of toxicological effects and dose effectiveness of a composition or mixture of compositions for an individual or in a population; determination of how two or more different agents administered in a single treatment might interact so as to detect any of synergistic, additive, negative, neutral or toxic activity; performing pre-clinical and clinical trials by providing new criteria for pre-selecting subjects according to informative profile data sets for revealing disease status; and conducting preliminary dosage studies for these patients prior to conducting phase 1 or 2 trials. These gene expression panels may be employed with respect to samples derived from subjects in order to evaluate their biological condition.

A gene expression panel is preferably selected in a manner so that quantitative measurement of RNA or protein constituents in the panel constitutes a measurement of a biological

condition (such as a leukemic state) of a subject. In one kind of arrangement, a calibrated profile data set is employed. Each member of the calibrated profile data set is a function of (i) a measure of a distinct constituent of a gene expression panel and (ii) a baseline quantity. Further information regarding derivation, analysis, and comparison of gene expression profiles and gene expression panels are disclosed in U.S. Patent Publication 2004/0133352 (Bevilacqua et al.), filed Nov. 8, 2002, and U.S. Patent Publication 2004/0132050 (Monforte et al.), filed Jul. 16, 2003, which are incorporated herein by reference in their entirety.

The practice of the present invention can employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, electrophysiology, and pharmacology, that are within the skill of the art. Such techniques are explained fully in the literature (see, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); DNA Cloning, Vols. I and II (D. N. Glover ed. 1985); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Transcription and Translation* (Hames et al. eds. 1984); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller et al. eds. (1987) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); *Scopes, Protein Purification: Principles and Practice* (2nd ed., Springer-Verlag); and *PCR: A Practical Approach* (McPherson et al. eds. (1991) IRL Press)).

Each of the following applications are incorporated herein by reference in their entirety, including all nucleic acid sequences, amino acid sequences, figures, tables, and claims: U.S. Provisional Patent Application Ser. No. 60/486,077, filed Jul. 9, 2003; U.S. patent application Ser. No. 10/263,419, filed Oct. 3, 2002; U.S. Provisional Patent Application Ser. No. 60/327,140, filed Oct. 3, 2001; U.S. Provisional Patent Application Ser. No. 60/334,277, filed Nov. 28, 2001; U.S. Provisional Patent Application Ser. No. 60/352,636, filed Jan. 28, 2002; U.S. Provisional Patent Application Ser. No. 60/412,450, filed Sep. 20, 2002; U.S. patent application Ser. No. 09/914,508, filed Nov. 7, 2001 (which is a National Stage Application of International Application Number PCT/US01/16408, filed May 21, 2001); U.S. Provisional Patent Application Ser. No. 60/277,700, filed Mar. 21, 2001; and U.S. Provisional Patent Application Ser. No. 60/209,245, filed Jun. 5, 2000.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for the purpose of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

## EXAMPLES

The examples presented herein can be summarized as follows. The data disclosed herein demonstrate that Dexter cultures consist of only three cell types: macrophages (~35%), hematopoietic cells (~5%), and nonhematopoietic cells (~60%). Using a percoll gradient centrifugation technique, the nonhematopoietic mesenchymal progenitor cells were isolated, free of macrophages and hematopoietic cells. A variety of techniques were used to identify the isolated cells as a multi-differentiated mesenchymal cell lineage co-expressing genes specific for multiple mesenchymal cell lineages including adipocytes, osteoblasts, fibroblasts and muscle cells.

Evidence that this multi- or pluri-differentiated mesenchymal progenitor cell is capable of supporting hematopoiesis is shown by the expression of a number of hematopoietic growth factors and extracellular matrix receptors. The SCID mouse experimental data provides evidence that since the MPCs can be purified to near homogeneity (95%) with relative ease, MPCs can be of value for enhancing engraftment of hematopoietic stem cells and bone marrow transplants. Additionally, increased survival rate in the SCID mouse model indicates that isolated MPCs can also be useful for the treatment of GvHD. An example of the administration of bone marrow cells and MPCs to breast cancer patients treated with chemotherapy is also provided.

A stepwise genomics strategy and an example of the genomic changes observed in leukemia associated MPCs is also provided. Cluster analysis was performed to show gene expression patterns in isolated MPCs of a normal individual and individuals with different leukemic conditions. The approach presented provides the basis for a new more objective means to diagnose patients suffering from leukemic conditions.

#### Example 1

##### Isolation and Characterization of MPCs from Dexter-Type Bone Marrow Stromal Cell Culture Systems

##### Bone Marrow Culture

Bone marrow samples were obtained from posterior superior iliac crest under general anesthesia for standard marrow transplantation. Marrow stromal cell cultures were set up using the residual cells recovered from the filters of Fenwal Bone Marrow Collection System after complete filtration, of the marrow samples. The filters were rinsed with phosphate-buffered saline without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (PBS-CMF). The cell suspension was subjected to Ficoll gradient isolation of the mononuclear cells (bone marrow MNCs). The bone marrow MNCs were washed ( $\times 2$ ) in PBS-CMF and suspended in McCoy's 5A with HEPES medium containing 12.5% fetal bovine serum (FBS), 12.5% horse serum, 1  $\mu\text{M/L}$  hydrocortisone and 1% penicillin/streptomycin (for this study McCoy's complete medium) and cultured under standard stromal-cell culture conditions (FIG. 1) (Seshi, et al. Blood 83, 2399 (1994) and Gartner, et al. Proc Natl Acad Sci USA 77, 4756 (1980). After two weeks, confluent stromal cell cultures were trypsinized (first passage), followed by splitting each T75 flask into two T150 flasks.

##### Morphologic and Phenotypic Characteristics of MPCs as Uncovered by Staining for Representative Mesenchymal Cell Lineage Markers

Two weeks after the first passage (above), confluent stromal cells were again trypsinized. Cytospins were prepared using aliquots of unfractionated cells for performance of various cytological, cytochemical and immunocytochemical stains.

Reactivity patterns of the bone marrow culture cells are outlined in Table 1. FIGS. 4A-E illustrate morphologic and phenotypic characteristics, as uncovered by staining for representative cell lineage markers. As illustrated in Table 1 and FIGS. 3 and 4A, Wright-Giemsa staining revealed three morphologically identifiable cell populations in Dexter type stromal cell cultures, macrophages, hematopoietic cells, and non-hematopoietic cells (labeled 4, 3, and 5, respectively).

The identity of macrophages was confirmed by immunostain using anti-CD68 antibody (FIG. 4B) and cytochemical stains for acid phosphatase and Sudan black. The identity of hematopoietic cells (including macrophages) was confirmed by immunostain using anti-CD45 antibody (FIG. 4C).

The remaining nonhematopoietic cells stained intensely positive for Periodic acid-Schiff, which was diastase sensitive, signifying the presence of large stores of glycogen (FIG. 4D). The presence of glycogen (6) was confirmed by electron microscopy (see FIG. 5). In this respect, MPCs are reminiscent of the glycogen-laden reticular cells in the developing bone marrow of human fetuses (observed by L.-T. Chen, L. Weiss, Blood 46, 389 (1975)). Glycogen deposition is viewed to be a developmentally regulated process during morphogenesis (H. Ohshima, J. Wartiovaara, I. Thesleff, Cell Tissue Res. 297, 271 (1999)).

In terms of lineage markers, up to 100% of the nonhematopoietic cells expressed two fat cell markers (Nile Red (FIG. 4E) and Oil Red O); an osteoblast marker (alkaline phosphatase (FIG. 4F)); and two fibroblast markers (fibronectin (FIG. 4G) and prolyl-4-hydroxylase). Greater than 85% of the nonhematopoietic cells were also positive for a muscle marker, actin (FIG. 4H). There was no evidence of expression of endothelial cell differentiation, as judged by immunohistochemical staining for CD34 and CD31 (data not shown).

The results indicate that the nonhematopoietic cells of the Dexter cultures are in fact a single, pluri-differentiated cell type co-expressing multiple mesenchymal cell lineage markers. The pluri-differentiated mesenchymal progenitor cells reported here are to be distinguished from the pluri-potential, but undifferentiated, MSCs that are generated in the absence of hematopoietic cells, such as in Friedenstein-type cultures.

TABLE 1

Reactivity patterns of bone marrow stromal cells based on cytological, cytochemical and immunocytochemical stains*, ***				
Figure	Test Utilized	Macrophages	Hematopoietic Cells	Mesenchymal Progenitor Cells
1 3 and 4A	Wright-Giemsa (Harleco)	Large cells with a small round nucleus & foamy cytoplasm: 35% of total cells	Small cells with minimal amount of cytoplasm: 5% of total cells	Large cells with a relatively irregular nucleus & cytoplasm compartmentalized into ectoplasm and endoplasm: 60% of total cells
2 4D	Periodic acid-Schiff (PAS) (Sigma)	0	0	~100% MPCs: staining restricted to ectoplasm in a ring-like fashion;

TABLE 1-continued

Reactivity patterns of bone marrow stromal cells based on cytological, cytochemical and immunocytochemical stains*, ***				
Figure	Test Utilized	Macrophages	Hematopoietic Cells	Mesenchymal Progenitor Cells
3 4C	CD45 (Dako, PD7/26 & 2B11)	100% macrophages (MΦ)	100% HCs	and completely abolished by diastase digestion 0
4 4B	CD68 (Immunotech, clone PG-M1)	100% MΦ	0	0
5	Sudan Black (Sigma)	~100% MΦ	0	0
6	Acid phosphatase (Sigma Kit No. 387)	100% MΦ; positive granules packed throughout cytoplasm	0	100% MPCs; positive granules in moderate amounts; staining restricted to endoplasm
7 4E	Nile Red (Sigma)	0	0	~100% MPCs: staining restricted to endoplasm
8	Oil Red O (Sigma)	0	0	~100% MPCs: staining restricted to endoplasm
9 4F	Alkaline phosphatase (Sigma Kit No. 85)	0	0	~100% MPCs: variable number of positive granules; staining restricted to endoplasm & plasma membrane**
10 4G	Fibronectin (Immunotech, clone 120.5)	0	0	~100% MPCs: staining restricted to endoplasm
11	Prolyl-4-hydroxylase (Dako, clone 5B5)	0	0	~100% MPCs: staining preferentially in the endoplasm
12 4H	Muscle actin (Ventana, clone HUC 1-1)	0	0	>85% MPCs: variable staining restricted to ectoplasm

\*The lineages of the markers tested above are: 3, hematopoietic cell marker; 4, 5 and 6, monocyte/macrophage markers; 7 and 8, adipocyte markers; 9, osteoblast marker; 10 and 11, fibroblast markers; 12 muscle marker.

\*\*One earlier study (Simmons, et al., Nature 328, 429-432) interpreted the localization of alkaline phosphatase staining as confined to the plasma membrane when in fact it is predominantly present within the endoplasm (compare FIG. 1C of this reference with FIG. 4F).

\*\*\* While well-accepted mesenchymal lineage markers were used, these markers do not necessarily lend themselves to simultaneous assessment of the same cell. For example, muscle-specific actin antibody worked only on formalin-fixed, paraffin embedded material, whereas stains like alkaline phosphatase, Oil Red and Nile Red are not anti-body based and involve varying fixing and staining conditions. Thus, the evidence shows that close to 100% of members of a morphologically distinct population # express multiple lineage markers of interest.

#### Bone Marrow Mesenchymal Progenitor Cell (MPC) Purification

To further investigate the characteristics of the MPCs, the nonhematopoietic stromal cells were then purified from the macrophages (~95% pure), the dominant "contaminating" cell type using the following method. Confluent monolayers of stromal cells resulting from first passage, above, were washed for three minutes in Ca<sup>2+</sup>/Mg<sup>2+</sup> free Hanks' balanced salt solution. Cells were incubated at room temperature for 45 minutes with intermittent mixing in serum-free McCoy's medium containing 10 mM L-leucine methyl ester (LME, Sigma). LME is a lysosomotropic agent that selectively kills and detaches macrophages. The detached macrophages were

removed by washing the monolayers twice in McCoy's complete medium, followed by trypsinization of the monolayers. The resulting single cell suspensions were fractionated by discontinuous Percoll gradient (70%, 50%, 30%, 20%, 10%) centrifugation at 800xG for 15 minutes at 4° C. in a fixed angle rotor (Avanti-J25 Beckman centrifuge) (FIG. 2). Low-density cells representing the macrophages resistant to detachment by LME separate as a band at the interface of serum and 10% Percoll and were discarded (1). High-density nonhematopoietic cells representing MPCs form a layer in the region of 30-50% Percoll (2). These were collected and washed twice by centrifugation through PBS-CMF. This protocol is conservatively expected to yield, >2.5x10<sup>6</sup> MPCs per

T-150 flask (i.e.,  $>50 \times 10^6$  MPCs per batch of 20 flasks). The purity of these preparations, typically about 95%, was routinely monitored by Wright-Giemsa staining.

#### Northern Blotting

Additional sets of multiple mesenchymal lineage markers were assessed by Northern blotting to eliminate any observer bias that might be inherent in morphological assessment. FIGS. 6A-M represent different gene probes used for hybridization. The sources of the gene probes employed and the major transcripts observed are outlined in the brief description of the figures.

Total RNA was prepared by dissolving the high-density cell pellets in Trizol (Life-Technologies). Total RNA samples from unfractionated stromal cells and BM MNCs were similarly prepared. The RNA samples were electrophoresed in a standard 1% agarose gel containing 2% formaldehyde in MOPS/EDTA buffer and blotted onto Immobilon-Ny+membrane. Probes were labeled using Prime-A-Gene Kit (Promega)—and  $\alpha^{32}\text{P}$  dCTP (NEN). Hybridization was performed at 65° C. in modified Church's hybridization solution using  $3 \times 10^6$  counts/ml in 10 ml (Millipore, 1998).

In FIGS. 6A-M, Northern blot analysis was performed side-by-side on fractionated stromal cells, non-hematopoietic cells freed of macrophages, and initial bone marrow mononuclear cell samples. Lanes 1 and 2 represent total RNA samples (10  $\mu\text{g}$  each) from unfractionated stromal cells (subjects S1 and S2, respectively). Lanes 3 and 4 represent total RNA samples (10  $\mu\text{g}$  each) from purified stromal MPCs (subjects S1 and S2, respectively). Lanes 5 and 6 represent total RNA samples (10  $\mu\text{g}$  each) from bone marrow mononuclear cells, the starting cells for bone marrow cell cultures (subjects S3 and S4, respectively).

The large transcripts, especially of collagen (lane 1, FIG. 6G) and fibronectin (lane 1, FIG. 6J), in RNA extracted from unfractionated stromal cells of subject 1 showed difficulty migrating into the gel. This observation correlates with the presence of an artifact of unresolved positive material in lane 1, FIG. 6A. Since the RNA extracted from unfractionated stromal cells of the subject 2 did not present this problem (lane 2, FIG. 6G, FIG. 6J and FIG. 6A), the observation does not impact on the overall interpretation of the results (see text). The lineages of markers tested were: monocyte/macrophage markers, CD68 and cathepsin B; adipocyte marker, adipsin; osteoblast markers, osteoblast-specific cadherin-11, chondroitin sulfate proteoglycan 2, collagen type I alpha 1 and decorin; fibroblast marker, fibronectin; muscle markers, caldesmon and transgelin. Marker signals were normalized to the amount of RNA loaded, which was based on densitometry of the GAPDH signals on the corresponding blot (Bio-Rad Model GS-700 Imaging Densitometer). Attenuation or enhancement of the marker signals in the purified stromal MPCs (i.e., lanes 3 and 4) relative to unfractionated stromal cells (i.e., lanes 1 and 2, respectively) is shown as fold A (decrease/increase) underneath the lanes 3 and 4; ND, means not determined.

The purity of the nonhematopoietic cells was demonstrated by a near complete absence of two macrophage markers, CD68 and cathepsin B (as shown by Northern blotting data, FIGS. 6A and 6B). As a positive control, bone marrow mononuclear cells rich in myelomonocytic cells abundantly expressed CD68 (lanes 5 & 6, FIG. 6A). The Northern blot results are consistent with a purity estimate of ~95% (vs. 60% in unfractionated samples) based on morphology and immunocytochemical staining for CD68.

Compared to unfractionated cells, the purified nonhematopoietic cells expressed significantly higher levels of markers representing fat cells (adipsin, FIG. 6D); osteoblasts (osteoblast-specific cadherin-11, chondroitin sulfate, collagen type

1 and decorin, FIGS. 6E-H); fibroblasts (fibronectin, FIG. 6J); and smooth muscle cells (caldesmon and transgelin, FIGS. 6K-L). No trace of osteoblast, fibroblast, or smooth muscle cell markers were detected in the bone marrow mononuclear cells, suggesting a less than detectable level of stromal cells or their precursors in bone marrow mononuclear cells. However, the fat cell marker, adipsin, was detected in all samples including the bone marrow mononuclear cells.

Taken together, the morphologic, cytochemical and immunocytochemical results (FIGS. 4A-H and Table 1), and the Northern blotting data (FIGS. 6A-M) indicate that the non-hematopoietic stromal cells of the Dexter cultures co-express markers specific for at least four different mesenchymal cell lineages.

This finding is especially intriguing because pluri-differentiation is often a feature of neoplastic cells (Brambilla and Brambilla, *Rev. Mal. Respir.* 3,235 (1986); Pfeifer et al., *Cancer Res.* 51, 3793-3801 (1991); Tolmay et al., *Virchow's Arch* 430, 209-12 (1997). However, a cytogenetic analysis of the Percoll-gradient purified MPCs showed a normal GTW banding pattern.

#### RT-PCR Analysis for Expression of Representative Hematopoietic Growth Factors and Extracellular Matrix Receptors by MPCs

RT-PCR was conducted in a total reaction volume of 100  $\mu\text{l}$  using 2  $\mu\text{g}$  each of total RNA; corresponding primers; and a master mix of the PCR reagents. The RT conditions included sequential incubations at 42° C. for 15 minutes, 99° C. for five minutes, and 5° C. for five minutes. The PCR conditions included: initial melting at 94° C. for four minutes; and cyclical melting at 94° C. for 45 seconds, annealing at 55° C. for 45 seconds and extension at 72° C. for 45 seconds with 34 cycles. PCR was terminated after final extension at 72° C. for ten minutes. Reaction products (G-CSF, SCF, each 25  $\mu\text{l}$ ; VCAM-1, ALCAM, each 50  $\mu\text{l}$ ; ICAM-1, 75  $\mu\text{l}$ ) were concentrated as necessary; electrophoresed along with a 100-bp DNA ladder (GIBCO-BRL) in a standard agarose (1%) gel in TAE buffer; and stained with ethidium bromide.

PCR products, shown in FIG. 7 lanes labeled 1-2, were generated using aliquots of the same RNA samples from purified stromal MPCs, as used for Northern blotting shown under FIG. 6 lanes 3 and 4, respectively. The gene transcripts amplified were as follows: G-CSF (granulocyte-colony stimulating factor); (Tachibana et al., *Br. J. Cancer*, 76, 163-74 (1997); SCF (stem cell factor, i.e., c-Kit ligand); (Saito et al., *Biochem. Biophys. Res. Commun.*, 13, 1762-69 (1994); ICAM-1 (intercellular adhesion molecule-1, CD54) and VCAM-1 (vascular cell adhesion molecule-1, CD106) (primers from R&D); and ALCAM (activated leukocyte cell adhesion molecule, CD166) (Bruder et al., *J. Bone Miner. Res.*, 13, 655-63 (1998)).

The observed PCR products for G-CSF (600 bp, i.e., the top bright band) and ALCAM (175 bp) were significantly different from the expected sizes (278 bp; 372 bp, respectively). However, sequencing of the gel-purified PCR bands and subsequent BLAST search showed a 99-100% identity with respective members. Attempts to detect c-Kit (i.e., SCF receptor) using primers as described (Saito et al., *Biochem. Biophys. Res. Commun.*, 13, 1762-69 (1994)) amplified a PCR product of ~300 bp with no homology to c-Kit (data not shown). The observed product sizes for SCF (about 730 bp); ICAM-1 (about 750 bp); and VCAM-1 (about 500 bp) were as expected.

As illustrated in FIG. 7, RT-PCR analysis showed that purified, multi-differentiated MPCs express both critical hematopoietic growth factor/cytokines, such as G-CSF and

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SCF as well as matrix receptors/hematopoietic cell adhesion molecules, i.e. ICAM-1, VCAM-1, and ALCAM.

## Example 2

Comparison of the Ability to Support In vitro  
Hematopoiesis by Purified MPCs vs. Unfractionated  
Bone Marrow Stromal Cells

CD34+positive cells (hematopoietic progenitor cells) were purified (Dyna kit) and cocultured with irradiated stromal monolayers for five weeks, followed by performance of standard colony assays for hematopoietic progenitors using methylcellulose medium supplemented with colony stimulating factors (using MethoCult medium from Stem Cell Technologies, Inc, Canada). Unfractionated bone marrow stromal cells and purified MPCs were prepared in the same manner as in Example 1. Data in FIG. 8 represents results from three experiments. Purified MPC provides increased preservation of hematopoietic progenitor cells compared to unfractionated stromal cells.

## Example 3

Animal Model for Enhanced Engraftment Capacity  
of MPCs

The Severe Combined Immunodeficiency Disease (SCID) mouse model is an ideal system in which to investigate MPC function. Engraftment of human hematopoietic progenitors in SCID mice requires either coadministration of exogenous human cytokines, or cotransplantation of human bone marrow plugs or bone fragments.

There has been discovered a convenient, new source for human bone marrow stromal cells for enhancing transplantation that does not require cytokines, bone fragment, or marrow. Unlike prior methods, the isolated cells of the present invention support human hematopoiesis in the SCID mouse model as effectively as whole marrow stroma. The transplantation of human marrow mononuclear cells combined with purified MPCs results in dramatically vigorous engraftment of human cells in spleen, bone marrow, liver, pancreas, lungs, stomach, and paravertebral neuronal ganglia of SCID mice. By contrast, mice receiving human bone marrow mononuclear cells alone or MPCs alone expectedly showed no detectable evidence of human hematopoietic cell engraftment. Also notably, the mortality rate was highest in mice that received unfractionated whole marrow stroma whereas purified MPC increased the survival rate which can be due to reduction in GvHD.

Transplantation of Human Cells in SCID Mice

Homozygous CB-17 scid/scid mice, six to eight weeks of age, were used. Lyophilized anti-asialo GM1 rabbit antibody (Wako Chemicals) was suspended in 1 ml sterile ddH<sub>2</sub>O, followed by pretreatment of mice with an IP injection of 20 ml (600 mg) ASGM1 antibody (to specifically deplete mouse macrophages and NK cells). Alternatively, one could use NOD/SCID mice lacking NK cell function, however, in light of highly promising preliminary results it was elected to continue use of scid/scid mice. The antibody treatment schedule included four-hour pre-engraftment and every seven days thereafter for the duration of the experiment. On the day of transplantation, the mice were irradiated with 200 or 300 cGy gamma-irradiation from a <sup>137</sup>CS source. Approximately 2.5×10<sup>6</sup> MPCs suspended in 0.5 ml McCoy's medium and/or 25×10<sup>6</sup> MNCs suspended in 0.2 ml were injected per mouse, intraperitoneally. Hematopoietic cell engraftment was

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assessed after five weeks by harvesting and analyzing representative hematopoietic and nonhematopoietic organs including blood, spleen, bone marrow (from two femurs and tibia) from euthanized mice.

5 Flow Cytometric Evidence

FIGS. 9A and 9B are flow cytometric evidence of human hematopoietic cells in a SCID mouse cotransplanted with marrow MPC. FIG. 9A shows the presence of CD45+/CD34+ progenitors in the marrow. FIG. 9B shows CD45/CD34-mature hematopoietic cells circulating in the mouse's blood.

10 Photomicrographs of Cells

FIGS. 10A-H shows engraftment of human hematopoietic cells in a SCID mouse cotransplanted with the purified marrow MPCs of the present invention. FIG. 10A shows a serial section of a mouse spleen stained with H & E. FIG. 10B shows a serial section of a mouse spleen stained with immunoperoxidase stain for CD45. FIG. 10C shows bone marrow stained for CD45. FIG. 10D shows a serial section of the mouse liver stained with H&E depicting involvement of periportal areas. FIG. 10E shows a serial section of the mouse stomach stained with H&E showing transmural infiltration. FIG. 10F shows a serial section of the mouse lung stained with H&E showing involvement of peribronchial area. FIG. 10G shows a serial section of the mouse pancreas stained with H&E. FIG. 10H shows a serial section of the mouse paravertebral ganglia stained with H&E.

FIG. 11A is a photomicrograph of a serial section of the spleen of a normal BALB/C mouse showing white pulp populated by darkly staining lymphocytes (H&E). FIG. 11B is a photomicrograph of the spleen of a SCID mouse showing white pulp largely consisting of lightly staining stromal framework (H&E). FIG. 11C is a photomicrograph of the spleen of a SCID mouse cotransplanted with human bone marrow MNC and the purified bone marrow MPCs of the present invention showing homing (engraftment) of human B cells to white pulp.

Southern Blotting Data

Hybridization of sample DNA using a DNA probe specific for human chromosome 17 alpha satellite DNA (p177H8) shows linear signal intensity with a 2.7 Kb band (arrow; autoradiogram exposed for only 45 minutes) (FIG. 12A). Lanes 1-contain human DNA starting 1000 ng to 100 ng admixed with 0 ng 900 ng of mouse DNA, total amount DNA loaded in each lane being 1 ug, allowing construction of a standard curve. The reported limit of detection with this technique is 0.05% human cells, which is more reliable than flow cytometry in detecting very low levels of human cell engraftment.

FIG. 12B is a Southern blot of EcoR1 digest of thymic genomic DNA from SCID mice. Lanes 1-5 were loaded with 500 through 100 ng human DNA. Lanes 6, 9-11 were loaded with DNA from mice which received unfractionated bone marrow stroma plus bone marrow mononuclear cells. Lanes 7, 8, 14, 15 were loaded with DNA from mice that received MPCs plus bone marrow mononuclear cells. Lanes 12, 13, were loaded with DNA from mice that received bone marrow mononuclear cells only. There is evidence of human cell engraftment in the mouse thymus in lanes 9 and 11 and lanes 14 and 15 evidenced by the 2.7 Kb band. There was no evidence of engraftment in mice that only received only bone marrow mononuclear cells, lanes 12 and 13.

FIG. 12C is EcoR1 digest of Lymph Node genomic DNA from SCID mice. Lanes 1-5 were loaded with 500 through 100 ng human DNA. Lanes 6, 9-11 were loaded with DNA from mice which received unfractionated bone marrow stroma plus bone marrow mononuclear cells. Lanes 7, 8, 14, 15 were loaded with DNA from mice that received MPCs plus



bone marrow mononuclear cells. Lanes 12, 13 were loaded with DNA from mice that received bone marrow mononuclear cells only. While there was evidence of engraftment of human cells in the mouse lymph nodes for mice that received unfractionated bone marrow stromal cells and MPCs, there was no evidence of engraftment in mice that only received only bone marrow mononuclear cells, lanes 12 and 13.

#### Increased Survival and Evidence of MPC Effect on GvHD

Increased Survival and Evidence of MPC Effect on GvHD: FIGS. 13A-1, 13A-2, 13B-1, and 13B-2 show graphs comparing the survival rate and engraftment of human hematopoietic cells in SCID mice cotransplanted with the purified bone marrow MPCs of the present invention versus unpurified bone marrow stromal cells. Mice in FIGS. 13A-1 and 13A-2 received 300 cGy irradiation dose and mice in FIGS. 13B-1 and 13B-2 received 200 cGy of irradiation. FIGS. 13A-1, 13A-2, 13B-1, and 13B-2 show comparable engraftment of human hematopoietic cells in SCID mice cotransplanted with purified MPCs versus unpurified bone marrow stromal cells and the markedly enhanced survival of mice receiving purified MPCs. Notably, no engraftment was observed in mice receiving bone marrow mononuclear cells alone.

The highest mortality rate, FIGS. 13B-1 and 13B-2, was observed in mice receiving the unpurified stromal cells and the bone marrow mononuclear cells. The increased mortality observed can be related to the presence of highly immunogenic macrophages and consequent GvHD. The mice with the highest survival rate, as shown in FIGS. 13A-1 and 13A-2, were the mice receiving purified MPCs and bone marrow mononuclear cells.

FIGS. 14A-D demonstrate apoptosis by TUNEL assay in organs of SCID mice that died after transplantation with human bone marrow mononuclear cells and unpurified bone marrow stromal cells. FIG. 14A shows a serial section of the liver of the mouse that survived. FIG. 14B shows a serial section of the liver of the mouse that died. FIG. 14C shows a serial section of the spleen of the mouse that survived. FIG. 14D shows a serial section of the spleen of the mouse that died. Hematoxylin counterstain was applied to sections in FIG. 14A and FIG. 14C. Methylgreen counterstain was applied to sections in FIG. 14B and FIG. 14D.

Notably, there is discrete TUNEL-positive nuclei in the liver of the expired mouse in FIG. 14B and complete absence of staining in the liver of the surviving mouse FIG. 14A. While some ill-defined globules of staining are observed in the spleen of the mouse that survived, the nuclear integrity of most of the cells is well preserved suggesting minimal or no apoptosis (FIG. 14C). By contrast, the dead mouse spleen (FIG. 14D) showed extensive TUNEL positivity precluding accurate interpretation. Control mouse liver and spleen showed results similar to those of the mouse that survived.

The size of the spleens from the mice that survived and the mice that died were compared. The dead mice were observed to have small and atrophic spleens correlating with lymphoid cell depletion and apoptosis.

The above results indicate that purified MPC can support human hematopoiesis in SCID mice as effectively as whole marrow stroma. Equally important is that the purified MPCs increased the survival rate. Evidence suggests that the increased survival can be due to a reduction in GVHD.

#### Administration of Bone Marrow Cells and Mesenchymal Progenitor Cells to Breast Cancer Patients Treated with Chemotherapy

A breast cancer patient undergoes a diagnostic posterior iliac crest bone marrow aspiration and biopsy using a local anesthetic. A small portion (2 to 3 ml) of the aliquot (10 to 20 ml) of marrow is submitted for routine histologic testing and determination of the presence of tumor cells using immunoperoxidase testing. The remainder of the cells are Dexter cultured for MPCs as described above in Example 1.

The patient also undergoes placement of a pheresis central venous catheter, and receives subcutaneous injections of G-CSF (filgrastin) 10 µg/kg/day as described in Peters, et al, Blood, Vol. 81, pgs. 1709-1719 (1993); Chao, et al, Blood, Vol. 81, pgs. 2031-2035 (1993); Sheridan, et al, The Lancet, Vol. 2, pgs. 891-895 (1989); and Winter, et al, Blood, Vol. 82, pg. 293a (1993). G-CSF injections begin at least three days before the first pheresis is initiated. G-CSF therapy is withheld if the white blood cell count rises above 40,000/L and is resumed once the white blood cell count drops to less than 20,000 µL.

If the patient is receiving only G-CSF as the vehicle for "mobilization" of peripheral blood progenitor cells, the patient must not have received chemotherapy within four weeks of the planned pheresis. If the patient has received both conventional chemotherapy and G-CSF treatment for mobilization, the patient must not have received chemotherapy within ten days of the planned pheresis, and the white blood cell count must be at least 800/µL and the platelet count at least 30,000 µL.

Daily pheresis procedures are performed using a Cobe Spectra instrument (Cobe, Lakewood, Col.), and each cellular collection is cryopreserved using a controlled-rate liquid nitrogen freezer, until at least  $15 \times 10^8$  mononuclear cells/kg are collected (Lazarus, et al., Bone Marrow Transplant, Vol. 7, pgs. 241-246 (1991)). Each peripheral blood progenitor cell is processed and cryopreserved according to previously published techniques. (Lazarus, et al., J. Clin. Oncol., Vol. 10, pgs. 1682-1689) (1992); Lazarus et al., (1991)).

Eight days before the patient is infused with the autologous peripheral blood progenitor cells, the patient receives chemotherapy over a period of 96 hours (four days), with the following chemotherapy agents: 1) Cyclophosphamide in a total dosage of 6 g/m<sup>2</sup> (1.5 g/m<sup>2</sup>/day for four days) is given via continuous intravenous infusion at 500 mg/m<sup>2</sup> in 1,000 ml normal saline every eight hours; 2) Thiotepa in a total dosage of 500 mg/m<sup>2</sup>/day for four days) is given via continuous intravenous infusion at 125 mg/m<sup>2</sup> in 1,000 ml normal saline every 24 hours; and 3) Carboplatin in a total dosage of 1,800 mg/m<sup>2</sup> (200 mg/m<sup>2</sup>/day for four days) is given via continuous intravenous infusion at 200 mg/m<sup>2</sup> in 1,000 ml of 5% dextrose in water every 24 hours.

The patient also receives 500 mg of Mesna in 50 ml normal saline IV over 15 minutes every four hours for six days (144 hours), beginning with the first dose of cyclophosphamide.

At least 72 hours after the completion of the chemotherapy, the MPCs are harvested from the Dexter culture(s). MPCs are collected and purified as described in Example 1. Cells are resuspended at approximately 10<sup>6</sup> cells/ml, and injected slowly intravenously over 15 minutes to provide a total dosage of from 10 to about  $5 \times 10^6$  cells.

MPCs can also be frozen and thawed to use when needed. For example, unfractionated cells from a Dexter culture are frozen. Upon thawing the cells are plated for about two days.

The MPCs are then purified as in Example 1 above. The MPCs are then replated with serum or in a serum free media and can remain stable for up to six days.

The day after the patient receives the MPCs, the frozen autologous peripheral blood progenitor cells are removed from the liquid nitrogen refrigerator, transported to the patient in liquid nitrogen, submersed in a 37° C. to 40° C. sterile water bath, and infused rapidly intravenously without additional filtering or washing steps. GM-CSF in an amount of 250 µg/m<sup>2</sup> then is given as a daily subcutaneous injection, beginning three hours after completion of the autologous blood progenitor cell infusion. The GM-CSF is given daily until the peripheral blood neutrophil count exceeds 1,000/µL for three consecutive days.

#### Example 5

##### Genomic Changes Observed in Leukemia Associated MPCs

The following is one example of how normal hematopoiesis might be compromised in leukemic conditions. The cellular interactions that underlie leukemic bone marrow involve stromal cells, leukemia/lymphoma cells, and normal hematopoietic progenitors (including those of myelopoiesis, erythropoiesis and megakaryocytopoiesis). In addition to displacing normal hematopoietic cells, the leukemia/lymphoma cells can potentially cause direct damage to the hematopoietic supportive stromal cells by inducing unwanted gene expression profiles and adversely affecting the normal hematopoiesis. The cellular interactions can be schematized as:

Leukemia/lymphoma cells ↔ stromal cells → normal  
hematopoietic progenitors

The point of this scheme is that regardless of whether stromal cell lesions are primary or secondary to leukemogenesis, the normal hematopoietic function is invariably compromised in leukemic conditions, though different leukemias affect myelopoiesis, erythropoiesis and megakaryocytopoiesis differentially. Contrary to the prevailing notion (see Marini, F et al., Mesenchymal Stem Cells from Patients with Chronic Myelogenous Leukemia Patients can be Transduced with Common Gene Transfer Vectors at High Efficiency, and are Genotypically Normal, 42nd Annual Meeting of the American Society of Hematology, Dec. 1-5, 2000 Poster #665), there has been observed extensive and striking gene expression changes in leukemia-associated bone marrow MPCs by using high-resolution genomics. Therefore, one embodiment of the present invention is to use transplantation of tissue-culture expanded, purified normal MPCs to improve granulopoiesis, erythropoiesis and thrombopoiesis, in for example MDS (most of MDS patients do not die from blast transformation but from complications related to cytopenias, i.e., hematopoietic failure).

The studies targeted acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and multiple myeloma (MM), one case of each. The AML patient was a 57 year-old woman with 52% myeloblasts in the bone marrow with immunophenotype confirmed by flow cytometry and a karyotypic abnormality of 45, XX, -7(6)146, XX [6]. Together with morphology, the diagnosis was AML arising in a background of myelodysplasia. The CML patient was a 35 year-old man with 2% blasts in the bone marrow and karyotypic abnormalities of Philadelphia chromosome and BCR/ABL gene rearrangement. Together with morphology, the diagnosis was CML in chronic phase. The MM patient was a 61 year-old woman with a IgA myeloma. The serum IgA level was 2.4

g/dl and the marrow plasma cell count was 37%. None of the patients was treated prior to obtaining marrow samples used in this study, to avoid any therapy-induced changes complicating the disease-associated changes.

The leukemic samples consisted of marrow aspirates that remained unused after clinical diagnostic studies were performed. A bone marrow sample obtained from an adult healthy male who had consented to donate bone marrow for standard marrow transplantation was simultaneously studied. The normal bone marrow sample consisted of residual cells recovered from the filters after complete filtration of the marrow sample. Setting up of Dexter-type stromal cell cultures and isolation of MPC were as described in Example 1. The normal stromal cells were studied without and after stimulation with TNFα because TNFα (and IL-4) are regarded as negative regulators of hematopoiesis. Notably these cytokines, especially TNFα, are elevated in marrow plasma of patients with myelodysplastic syndromes (MDS), the clinical hallmarks of which are anemia, leukopenia and thrombocytopenia (i.e., pancytopenia). TNFα and IL-4 are considered possible mediators of hematopoietic dysregulation typical of MDS.

##### A Stepwise Genomics Strategy Encompassed

Preparation of total RNA from MPC samples → generation of cDNA → preparation of ds DNA → in vitro transcription into cRNA → fragmentation of cRNA → hybridization of target RNA to a microarray of known genes (Affymetrix genechip containing DNA from ~12,000 known human genes, e.g., U95A oligonucleotide microarray) → analysis of differentially expressed genes using an appropriate software (GENESPRING) to discern the patterns of gene expression or genomic signatures by a given MPC type.

##### Cluster Analysis Showing Gene Expression Patterns in Bone Marrow MPC Isolated from a Normal Individual and Patients with Different Leukemic Conditions

Genes with correlated expression across bone marrow MPC types: GENESPRING was used for cluster analysis. Prior to application of an agglomerative hierarchical clustering algorithm, microarray signals were normalized across experiments (i.e., from one MPC type to another) making the median value of all of measurements unity, so different experiments are comparable to one another. The signals were also normalized across genes in order to remove the differing intensity signals from multiple experimental readings. Genes that are inactive across all samples were eliminated from analysis. Notably, 7398 genes out of 12,626 genes (present on the Affymetrix genechip used) passed the filter of a normalized signal intensity of at least 0.1 across at least one of the five experiments performed. Cluster analysis was performed with standard correlation (same as Pearson correlation around zero) as the distance metric, a separation ratio of 0.5 and a minimum distance of 0.001 as provided by the software application. A closer relationship between CML- and MM-associated MPCs was observed, which in turn are related to AML-associated MPC, thus transforming global patterns of gene expression into potentially meaningful relationships.

Two-dimensional cluster analysis of tissue vs. gene expression vectors: A gene tree was constructed. Genes cluster near each other on the "gene tree" if they exhibit a strong correlation across MPC experiments and MPC tree branches move near each other if they exhibit a similar gene expression profile. The data indicated that the two-way clustering readjusted the location of a number of genes resulting in accentuation of genomic signatures of each cell type. Investigators can usefully catalog genes composing any unique or signature cluster of interest by creating a gene list and disclosing their identities.

Self-Organizing Map (SOM) Clusters (6x5) Show Differential Gene Expression in Bone Marrow MPC Isolated from Different Hematopoietic Conditions

Generation of SOM clusters involved prior normalization and filtering of the data. SOM algorithm was applied as provided by GENESPRING. Visualization of SOM clusters in combination with hierarchical clustering (i.e., MPC tree) revealed correlated meaningful patterns of gene expression. Predicated on the basis of SOM operating principle, the related SOM clusters tend to be located physically close to each other. For example, the juxtaposition of the SOM clusters with the common denominator containing genes that are up-regulated in AML/MDS-associated MPC. Whole or part of any SOM cluster can be selected to make a gene list providing the identities of the genes involved.

Genes Highly Expressed in Normal MPC but Absent or Minimally Expressed in Leukemia-Associated MPC

Lists of genes that are down-regulated in leukemia-associated MPC (AML/MDS, CML and MM) were created in comparison to normal MPC. A Venn diagram was made using these three gene lists. GENESPRING allows creation of sublists of genes corresponding to union, intersection and exclusion. Transcriptional profiles of any of these sublists of genes can be visualized across MPC samples of interest. The following is one such sublist of genes containing genes that are highly expressed in normal MPC and down-regulated in leukemia-associated MPCs revealing the identity of the subset of genes of interest: putative, wg66h09.x1 Soares *Homo sapiens* cDNA clone, *Homo sapiens* mRNA for CMP-N-acetylneuraminic acid hydroxylase, *Homo sapiens* cDNA clone DKFZp586G0421 (symptom: hute 1), Human mRNA for histone H1x, Putative monocarboxylate transporter *Homo sapiens* gene for LD78 alpha precursor, Interacts with SH3 proteins; similar to c-cbl proto-oncogene product, wg82b12.x1 Soares *Homo sapiens* cDNA clone, Human atrial natriuretic peptide clearance receptor (ANP C-receptor) mRNA, Human 71 kDa 2'5' oligoadenylate synthetase (p69 2-5A synthetase) mRNA, *Homo sapiens* hMmTRA1b mRNA, Human GOS2 protein gene, Preproenkephalin, Human guanylate binding protein isoform I (GBP-2) mRNA, Human gene for hepatitis C associated microtubular aggregate protein p44, 17-kDa protein, Human insulin-like growth factor binding protein 5 (IGFBP5) mRNA, GS3686, Human monoamine oxidase B (MAOB) mRNA, Insulin-like growth factor II precursor, Human insulin-like growth factor binding protein 5 (IGFBP5) mRNA, Similar to ribosomal protein L21, X-linked mental retardation candidate gene, and *Homo sapiens* mRNA; cDNA DKFZp434A202.

Genes not expressed in normal MPC but highly expressed in leukemia-associated MPC

Lists of genes that are upregulated (instead of down-regulated) in leukemia-associated MPCs (AML/MDS, CML and MM) were created in comparison to normal MPC and a Venn diagram was made. The following is one such sublist of genes containing genes that are inactive in normal MPC but up-regulated in leukemia-associated MPCs revealing the identity of the subset of genes of interest: Beta-tropomyosin, *Homo sapiens* clone 24659 mRNA sequence, Human mRNA for DNA helicase Q1, OSF; contains SH3 domain and ankyrin repeat, ym22b12.r1 Soares infant brain INIB *Homo sapiens* cDNA clone, Human mRNA for pre-mRNA splicing factor SRp20, Human mRNA for golgi alpha-mannosidase II, OSF-2os, *Homo sapiens* gene for Proline synthetase, hk02952 cDNA clone for KIAA0683, wi24g10.x1 *Homo sapiens* cDNA clone, Lysosomal enzyme; deficient in Sanfilippo B syndrome, CTP synthetase (AA 1-591), WD repeat protein; similar to petunia AN11, Human mRNA for 5'-terminal

region of UMK, complete cds, *Homo sapiens* chemokine exodus-1 mRNA, complete cds, Human GPI-H mRNA, complete cds, *Homo sapiens* mRNA encoding RAMP1, Transforming growth factor-beta-2 precursor, and *Homo sapiens* mRNA for KIAA0763 protein.

Visualizing Expression of Phenotypically & Functionally Relevant Genes across Samples of Normal & Disease-Associated BM MPC

Although GENESPRING is a highly flexible and user-friendly software application, it lacks the facility to create functionally relevant gene lists containing user-defined key words. This limitation was overcome by devising the following method via MICROSOFT EXCEL. A stepwise protocol to create such a gene list using EXCEL includes: Open the annotated microarray genome file (e.g., Affymetrix U95A) in EXCEL→select the column with gene names→select Data from pull-down menus Filter→AutoFilter→Customs enter key words (e.g., cell adhesion or cell cycle) OK→generates a new EXCEL worksheet with the list of genes containing the key words. Copy and paste the list of genes containing the key words into GENESPRING and save the gene list with a meaningful name. Twenty-two (22) such functionally relevant gene lists (Table 2) were created.

The resulting approach is a simple and powerful way to peer into the expression profiles of focused sets of functionally relevant genes across samples of interest. For example, the human vascular cell adhesion molecule-1 (VCAM-1) gene is completely down-regulated in AML/MDS and the human insulin-like growth factor binding protein (hIGFBP1) gene is up-regulated in AML compared to all other samples. Similarly, *Homo sapiens* gene for LD78 alpha precursor is down-regulated in all of leukemia-associated MPCs. Finally, the lineage markers CD45 and CD68 are essentially absent from the leukemia-associated MPCs attesting to the high degree of purity achieved by the sample preparation technique of the present invention.

## Results

The genomic changes observed in leukemia-associated MPCs are striking. As shown in Table 2, the changes (up-regulation and/or down-regulation) involved hundreds of genes. These changes were most dramatic in MPC associated with AML arising in a background of MDS and involved multiple classes of genes (Tables 1-2). Expectedly, the TNF $\alpha$ -induced changes were extensive. Given the high level of purity of MPC preparations, the enormous genomic changes observed are reflective of the underlying pathologic lesions in the MPCs themselves (and not due to the contaminating leukemic cells and/or macrophages). These studies strongly support the hypothesis that stromal cells in a leukemic patient are functionally defective and therefore purified MPCs are of value in restoring the loss of hematopoietic function in leukemic patients.

TABLE 2

Magnitude of global gene expression changes in leukemia-associated and TNF $\alpha$ -stimulated MPCs in comparison to normal MPC				
	AML/MDS MPC	CML MPC	MM MPC	TNF $\alpha$ MPC
# of genes up-regulated	234	112	108	279
# of genes down-regulated	379	208	251	164

TABLE 3

Functional classes of genes analyzed across  
normal and leukemia-associated MPCs

Annexins (14)
Caspases & apoptosis-related transcripts (33)
Cadherins (50)
Calmodulins/calmodulin-dependent kinases (25)
Cell adhesion molecules (20)
Cathepsins (19)
Collagens (71)
Cell division cycle-related transcripts (36)
Cytokines (19)
Epidermal growth factors and related transcripts (22)
Fibroblast growth factors (21)
Fibronectins (6)
Galectins (6)
Growth factors (136)
IGF system (24)
Interleukins/receptors (76)
Integrins/disintegrins (70)
Lineage-related markers (19)
Laminins (13)
Platelet-derived growth factors & receptors (12)
TNF alpha-related transcripts (29)
TGF beta-related transcripts (25)

The gene lists in Table 3 were created as described above and analyzed using GENESPRING. The numerical value in parenthesis refers to the number of transcripts in the corresponding class of genes analyzed.

#### Example 6

The present invention provides the following benefits: a) identification and documentation of BM stromal cell gene expression patterns under varied, normal, and leukemic hematopoietic conditions; b) identification of stromal cell genes that can be therapeutic targets for improvement of normal hematopoietic function that is constantly compromised in leukemic patients, and identification of similar targets for arresting the growth and progression of neoplastic clones since stromal cells provide the necessary support for preferential growth of leukemic cells (CLL, MM) within BM and protect the leukemic cells from chemotherapy-induced death (MM); and c) identification of new biological bases and new diagnostic markers for refinement of the classification and diagnosis of leukemia. This present invention can also lead to important insights into the pathogenesis of leukemia. In broad terms, analysis of global gene expression or transcriptome (transcriptional profile composed of all transcribed regions of the genome) is considered a nonbiased discovery-driven (as opposed to hypothesis-driven) approach to the analysis of gene expression. A stepwise genomic strategy encompasses preparation of total RNA from cells of interest, to generation of cDNA, to preparation of ds DNA, to in vitro transcription into cRNA, to fragmentation of cRNA, to hybridization of target RNA, to a microarray of known genes (and/or ESTs), to analysis of differentially expressed genes using an appropriate software to discern the patterns of gene expression or genomic signatures by a given disease-associated cell type.

Furthermore, the present invention can test the utility of sample preparation technology applied to normal EM-derived MPCs (untreated and treated with representative cytokines) and MPCs derived from patients with representative pre-leukemic and leukemic conditions for performance of high-resolution DNA microarray technology (Affymetrix genechip containing DNA from 12,000 known human genes, e.g., U95A oligonucleotide microarray).

Representative cytokines which are pathologically altered in hematopoietic conditions and that can be used in this study include TNF- $\alpha$ , TGF- $\beta$  and interferon- $\gamma$ . The pre-leukemic conditions include myelodysplastic syndromes (MDS) and the leukemic conditions include chronic myeloid leukemia (CML), acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and multiple myeloma (MM).

The front-end strategy of microarray analysis involves the use of Percoll-gradient purified MPCs. As a follow-up strategy, to validate the stromal cell origin of the differentially expressed genes, MPCs obtained from cytopins of BM stromal cells by laser-capture microdissection (LCM) selected on the basis of morphology (FIG. 3) are used followed by "real-time" quantitative polymerase chain reaction (PCR). This can be performed with an LGM system as well as a "real-time" QPCR system. Validation can be performed on at least one sample from each of 6 normal BM M7NC/MPC types and on one sample from each of 5 leukemia-associated MPC types. Validation is considered successful if the microarray results and PCR results on a given MPC sample match using a suite of 20 genes selected based on median pattern of microarray results for the given cell type. This approach not only validates the microarray results but also ascertains the stromal cell origin of the expressed genes.

The standard published protocols involving LGM and "real-time" quantitative PCR and the instructions accompanying the equipment are used for performing the experiments.

Stepwise LCMJ real-time QPCR protocol entails the following. Cytopins are made from BM stromal cells. The cytopins are stained with hematoxylin and MPC is selected for based on morphology. Microdissect up to 1,000 MPC from each sample. RNA is extracted and reverse transcribed into cDNA. The cDNA is amplified using gene-specific primers and "real-time" quantitative PCR.

By applying the combined power of different analytical techniques (such as hierarchical clustering and self-organizing maps) together with the recently developed sample preparation technology for stromal cells the present invention provides a molecular biological basis that can allow refinement of the classification and diagnosis of leukemias and lymphomas, uncovering the suspected disease heterogeneity. This enables the deciphering of the genomic expression profiles or signatures of bone marrow stromal cells in about 10 different physiologic states and about 20 different leukemic states. In addition to aiding in refinement of the classification and diagnosis of the hematopoietic malignancies, the data provides clues to potential novel drug targets and insights into pathogenesis.

The present invention functions by identifying the MPC genes that are differentially expressed after stimulation with different hematopoietic cytokines implicated in the pathogenesis of pre-leukemic conditions (MDS); in actual pre-leukemic disorders (MDS); and in overt leukemias (CML, AML, CLL, ALL, MM) as well as in lymphomas that have a leukemic phase with involvement of BM.

The present invention is accomplished by first determining the median gene expression profiles for MPCs associated with each disease and stimulated by each cytokine of interest (this objective can be achieved by treating the gene expression vectors of individual cases in each MPC category as replicates this capability is available in GENESPRING software application). Then the gene groups that are up regulated and down regulated and that are common to all the members in a given MPC category are identified (this is accomplished using a series of Venn diagrams and creating required gene lists via GENESPRING). Finally, the up-regulated and down-regulated gene sets for a given disease-associated or cytokine-

ine-stimulated MPC are combined. This allows the identification of gene sets with minimal number of elements that are unique to a given MPC type with a capability to discriminate one MPC type from another (this can also be accomplished by means of a series of Venn diagrams and lists of required genes obtained via GENESPRING). Such gene sets can be of immense diagnostic value as they can be routinely used in an assay simpler than microarray analysis (for example "real-time" quantitative PCR). Such gene sets can additionally provide insights into pathogenesis and possible targets for design of new drugs.

Determine expression profiles of MPC genes which are regulated as a result of exposure of normal MPCs to cytokines that are known to have a hematopoietic support role and/or are abnormally elevated in pre-leukemic/leukemic conditions, i.e., TNF  $\alpha$ ; IL-4; TNF  $\alpha$ +IL-4; interferon  $\gamma$ , TGF  $\beta$ ; PDGF; FGF; EGF; and calmodulin.

TNF  $\alpha$ ; IL-4 and IFN  $\gamma$  are potent negative regulators of hematopoiesis. Notably these cytokines, especially TNF  $\alpha$ , are elevated in marrow plasma of patients with myelodysplastic syndromes (MDS), the clinical hallmarks of which are anemia, leukopenia and thrombocytopenia (i.e., pancytopenia). TNF  $\alpha$  and IL-4 are thus possible mediators of hematopoietic dysregulation typical of MDS. Studies regarding these regulators can uncover the molecular pathways leading to cytopenias in MDS patients. As indicated earlier, myeloproliferative disorders are another, in some ways similar, group of hematopoietic disorders that are clonal in origin but not overtly malignant clinically. These MPDs include polycythemia vera, essential thrombocythemia, idiopathic myelofibrosis (agnogenic myeloid metaplasia) and chronic myelogenous leukemia. These disorders have the potential to change from one to the other at any time, however the signals that trigger such conversion remain enigmatic. Idiopathic myelofibrosis (IMF), in which stromal cells seem to play a profound pathogenetic role, is characterized by fibrosis of the marrow cavity, extramedullary hematopoiesis, splenomegaly, and anemia and leukoerythroblastic features in the peripheral blood. While myeloproliferation is known to be a clonal process, the accompanying stromal cell proliferation and fibrosis are believed to be a polyclonal reactive process that is likely to be due to increased intramedullary activity of a number of cytokines including TGF  $\beta$ , PDGF, FGF, EGF and calmodulin, as shown by other investigators.

Cancer genomics is a rapidly expanding area of investigation. The focus is unique however in emphasizing not the leukemic cells themselves but rather BM stromal cells that provide a haven to various types of pre-leukemia and leukemia cells, non-Hodgkin's lymphomas (NHLs) and metastatic cancers (METs). Pre-leukemic clonal neoplastic conditions include myelodysplastic syndromes (MDSs) and myeloproliferative disorders (MPDs). Stromal cells are known to produce and/or respond to growth factors such as EGF, PDGF, FGF, VEGF, and cytokines such as IL-1 or TNF  $\alpha$ , partially explaining the interactive relationship between stromal cells and cancer cells, especially in MDS and CML.

In spite of similarities between BM stroma and non-BM stroma, certain sharp distinctions do stand out. Non-BM stromal cells are terminally differentiated fibroblasts, while BM stromal cells represent a unique pluripotent or pluridifferentiated mesenchymal cell type, thus exhibiting preserved developmental "plasticity". Using 5-10 cc BM aspirate samples from adult leukemic patients and 3-5 cc BM samples from pediatric patients with ALL, the study can analyze the BM stroma. One cc of marrow sample can produce at least 1 T-150 flask of stromal cells. One concern is that it can be hard to obtain marrow samples from cases like CML and myelofi-

brosis. In such cases stromal cells are grown using peripheral blood samples as described in the prior art. At least one flask of stromal cells (i.e., 1 cc marrow) to yield the RNA required for analysis. About 10 cases of each type of leukemia/lymphoma were studied. The study provided important insights into the functioning of the BM microenvironment in normal and leukemic hematopoiesis.

A database including all of the above information and that can include age, gender and associated major illness in terms of clinical/pathologic diagnosis for each subject/patient can be created. This can also include information on cytogenetic, molecular and flow cytometric studies. Finally, also included can be the information on clinical course in terms of disease progression and response to treatment exercising adequate care to protect the identity of individual patients. The study analyzed genomic expression profiles or signatures of bone marrow stromal cells derived from about 12 different normal bone marrow states and about 19 different leukemia/lymphoma states, approximately 10 cases of each as shown in Table 4, accounting for a total of 310 samples.

Using the information of the present invention, those of skill in the art can: a) study select gene or sets of genes as relevant to hematopoietic disease conditions using relatively inexpensive but low-throughput technologies such as Northern blotting, RNase protection assays and/or PCR intended for gene expression analysis; b) reanalyze the primary data by using newer and more powerful bioinformatic tools as they become available; and/or c) identify newer drug targets and diagnostic markers relevant to specific diseases, such as MM or CML etc.

TABLE 4

Scope of human BM samples targeted for DNA microarray analysis (approximately 10 cases of each)	
Normal BM mononuclear cells (NMNC)	
Normal BM stromal cells, unfractionated and unstimulated (NBMS)	
Normal purified mesenchymal progenitor cells, unstimulated (NMPC)	
NMPC stimulated with 9 different cytokines:	
NMPC stimulated with TNF $\alpha$ (TNF $\alpha$ MPC)	
NMPC stimulated with TGF $\beta$ (TGF $\beta$ MPC)	
NMPC stimulated with interferon $\gamma$ (IFN $\gamma$ MPC)	
NMPC stimulated with IL-4 (IL-4 MPC)	
NMPC stimulated with TNF $\alpha$ + IL-4 (TNF $\alpha$ + IL-4 MPC)	
NMPC stimulated with PDGF (PDGF MPC)	
NMPC stimulated with EGF (EGF MPC)	
NMPC stimulated with FGF (FGF MPC)	
NMPC stimulated with calmodulin (calmodulin MPC)	
MDS - Refractory anemia (MDS-RA MPC)	
MDS - Refractory anemia with ringed sideroblasts (MDS-RARS MPC)	
MDS - Refractory anemia with excess blasts (MDS-RAEB MPC)	
MDS - Chronic myelomonocytic leukemia (M1)S-CMML MPC)	
MPD - Polycythemia vera (MPD-PV MPC)	
MPD - Essential thrombocythemia (MPD-ET MPC)	
MPD - Myelofibrosis (MPD-LMF MPC)	
CML (CML MPC)	
AML-M0/M1/M2 (AML-MOJM1JM2 MPC)	
AML-M3 (APL) (AML-M3 MPC)	
AML-M4/M5 (myelomonocytic) (AML-M4iM5 MPC)	
ALL-L1/L2 (lymphoblastic) (ALL-L1L2 MPC)	
ALL-L3 (Burkitt's) (ALL-L3 MPC)	
Multiple myeloma (MM MPC)	
CLLISLL (CLL/SLL MPC)	
Follicle center cell lymphoma (FCL MPC)	
Mantle cell lymphoma (MCL MPC)	
Lymphoplasmacytic lymphoma (LPL MPC)	
Marginal zone lymphoma (MZL MPC).	

#### Human Subjects

This study involves the use of bone marrow (BM) samples from human subjects. BM samples can be obtained from normal subjects (male and female 20-45 years) as well as

leukemic patients after informed consent is obtained. Leukemic cells can be obtained from diagnostic samples of BM of adult and pediatric patients (in those cases in which cells remain unused after clinical diagnostic studies are preformed; i.e., about 90% of cases).

#### Example 7

In broad terms, global gene expression analysis is considered a nonbiased discovery-driven (as opposed to hypothesis-driven) approach to the analysis of protein expression. A stepwise proteomics strategy encompasses: solubilization of proteins from cells of interest; 2-D gel electrophoresis (IPG DALT); staining and image analysis of gels; excision of protein spots of interest; trypsin digestion of proteins; mass spectrometry (MALDI-TOF MS and/or ESI MS/MS) performed on tryptic fragments; identification of proteins by database searching. The present invention provides a method to analyze the population of expressed proteins (i.e., proteome) of BM MPCs in relation to hematopoiesis in collaboration with a state-of-the-art mass spectrometry facility.

The large-format 2-D gel electrophoretic system is used for reproducible separation of MPC proteins and to prepare 2-D PAGE protein maps for normal bone marrow-derived MPCs (untreated and treated with representative cytokines, e.g., TNF alpha. and/or IL-4) and for MPCs derived from patients with representative pre-leukemic/premalignant and leukemic/malignant conditions. The pre-leukemic conditions include myelodysplastic syndromes (MDS) and the leukemic conditions include chronic myeloid leukemia (CML), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and multiple myeloma (MM). The protein samples can consist of culture supernatants/secreted proteins; extracellular matrix (ECM) proteins; plasma membrane proteins solubilized using a three-step differential extraction protocol, employing conditions of progressively increasing solubility; and whole cell lysate proteins similarly solubilized using the three-step differential extraction protocol. This subproteome approach not only simplifies the 2-D PAGE electrophoretic protein patterns but also reveals additional proteins, which would otherwise have gone undetected.

The system of the present invention can be used to differentially express MPC proteins (i.e., those that increased or decreased in intensity as compared to 2-D PAGE protein maps of normal, unstimulated MPCs) using mass spectrometry (MALDI-MS and/or nanoelectrospray ionization MS/MS) and/or Western blotting and/or Western-ligand blotting.

Using high-resolution proteomics with the added power of high-throughput robotics, enables the system to identify on a larger (semi-comprehensive) scale the MPC proteins that are differentially expressed in conditions that simulate pre-leukemic bone marrow (following stimulation with different cytokines); and in actual pre-leukemic disorders (MDS) as well as in overt leukemias (CML, AML, CLL, ALL, MM).

The system of the present invention enables the identification of MPC proteins whose expression is regulated as a result of exposure of normal MPCs to cytokines that are known to have a hematopoietic support role and/or are abnormally elevated in pre-leukemic/leukemic conditions, i.e., TNF  $\alpha$ ; IL-4; TNF  $\alpha$ +IL-4; interferon  $\gamma$ , TGF  $\beta$ ; PDGF; FGF; EGF; and calmodulin.

The system of the present invention also enables the identification of MPC proteins for which expression is altered as a result of exposure of normal MPCs to agents that are clinically used for mobilization of hematopoietic stem cells from

BM into peripheral blood to facilitate easy collection and subsequent transplantation, e.g., G-CSF and G-CSF plus cyclophosphamide.

Further, the system of the present invention enables the identification of Identify the MPC proteins whose expression is pathologically altered in hematopoietic disease states such as: MDS, CML, AML, CLL, ALL and MM by matching the 2-D PAGE protein maps of disease-associated MPCs with the 2-D PAGE database of normal MPCs. If a protein of interest does not exist in the normal MPC proteome, or if it exists in the normal MPC proteome but has not yet been characterized, then such proteins can be identified by MALDI-MS and/or Nano ESI MS/MS.

The system facilitates understanding of the pathogenetic mechanisms by identifying the phosphoproteins involved in cell signaling pathways. The systems immunoblots the whole cell lysate proteins of normal MPCs, untreated and treated with respective cytokines, using antibodies to phosphotyrosine, phosphoserine, and phosphothreonine. The system then locates the corresponding putative phosphoprotein spots on the gel and identifies the proteins by MALDI-MS and/or Nano ESI MS/MS. Similarly, the system can locate the altered phosphoproteins by immunoblotting the whole cell lysate proteins of untreated MPCs derived from leukemic patients. If a protein of interest does not exist in the normal MPC proteome, or if it exists in the normal MPC proteome but has not yet been identified, then MALDI-MS and/or Nano ESI MS/MS can identify the protein.

Bone Marrow MPCs Derived from a Leukemia Background Express Distinctively Different Patterns of Cell Adhesion Molecules from Normal MPCs

BM stromal cells provide the background required for homing and subsequent proliferation and differentiation of hematopoietic stem cells. BM stromal cells also provide a rich microenvironment for metastases and growth of various leukemias. Based on the hypothesis that homing of normal hematopoietic cells and leukemic cells to marrow utilize the same adhesion mechanisms, it was questioned whether there are fine regulatory distinctions in terms of quantitative differences in the expression of the adhesion molecules in normal vs. leukemic BM microenvironments. In a pilot study 11 cell adhesion molecules (CAMs) and several lineage-associated markers for Northern blot analysis were targeted. Dexter-type cultures were grown under standard stromal cell culture conditions using bone marrow samples from a normal individual and from one patient diagnosed with and treated for acute myelogenous leukemia (AML). Representative cultures were treated with cytokines such as TNF  $\alpha$  alone, IL-4 alone, and TNF  $\alpha$  plus IL-4. MPCs from unstimulated and cytokine-treated cultures were purified using Percoll gradient techniques disclosed above. Total RNAs were extracted by a standard method and analyzed by Northern blotting. This study demonstrated expression by MPCs of several CAMs, heretofore unsuspected of expression by BM stroma. These include an embryonic endothelial cell protein Dell (developmental endothelial locus 1), galectin-I, human milk fat globule protein (RMFG, lactadherin), and epithelial membrane protein 1 (EMP 1). Secondly, MPCs from the AML patient expressed significantly lower levels of mRNA for three CAMs Del-1, galectin-1, and collagen type 1 as well as for the adipocyte marker adipisin, and to a minor degree the muscle-associated protein caldesmon. On the other hand, mRNA for CAMs like TGF beta-inducible BiGH3, HMFG, osteoblast-specific cadherin 11, and VCAM1 were dramatically increased in AML-associated MPCs. CAMs such as integrin beta 5, fibronectin, EMP 1 and the muscle-associated molecule transgelin are variably increased in diseased MPCs and

appear to be unaffected by treatment with cytokines tested. ICAM I was undetectable at basal level in either patient or normal samples, but was slightly elevated by TNF  $\alpha$  and markedly elevated by TNF  $\alpha$  plus IL-4. VCAM1 was mildly up regulated by TNF  $\alpha$  alone or IL-4 alone, but markedly up regulated by TNF  $\alpha$ /IL-4 in combination. Also, the MPCs from the patient were much more sensitive to stimulation by these inflammatory cytokines than were the normal MPCs. These studies establish that stromal cells in a leukemic patient are functionally defective.

#### Role of Leptin Receptor in Hematopoiesis Using Human Marrow Stromal Cells as a Model

The receptor for the product of the obesity gene, leptin, is widely distributed in tissues ranging from central nervous system to reproductive system to hematopoietic system. Within hematopoietic system, OB-R is reportedly expressed on diverse cell types ranging from early CD 34+hematopoietic stem cells to circulating monocytes. Leptin acts on monocytes to induce production of TNF  $\alpha$  and IL-6, which are powerful regulators of hematopoiesis. However, literature reports on the expression of leptin or its receptor on stromal cells are infrequent. To date, one particular report suggests that leptin acts on the stromal cells to enhance their differentiation into osteoblasts and to inhibit differentiation into adipocytes. Because leptin is an adipocyte-generated hormone and because marrow stromal cells represent a unique pluridifferentiated mesenchymal cell type expressing some adipocytic features, the expression of the leptin receptor by these cells was investigated in the hope of revealing its role in hematopoiesis.

By Northern blotting marrow stromal cells showed abundant expression of OB-R, consistent with their adipocytic nature. In terms of regulation, exposure of the stromal cultures to different cytokines revealed an interesting pattern of OB-R. As shown, G-CSF and TNF  $\alpha$  down-regulated OB-R while IL-4 up-regulated OB-R expression by stromal cells. Simultaneous treatment of stromal cultures with TNF  $\alpha$  and IL-4 nearly abolished OB-R expression. The expression of OB-R was also analyzed at the protein level by a high-resolution, high capacity 2-D, PAGE system, followed by Western blotting.

More specifically, the method provides the identification of leptin receptor in human BM stromal cell membrane protein extracts using 2-D Western blotting. The expression of OB-R was investigated at protein level using 2-D PAGE, followed by Western blotting. Two isoforms differing in molecular weight of 2.2 kDa (60.2; 62) and an isoelectric point of 0.2 pH unit (5.78; 5.98, respectively) have been identified (the pH was determined by using the values specified by the IPG strip manufacturer). The ability to subsequently stain the same Western blot with gold stain allowed precise localization of the immunoreactive protein spots of interest on the blot. The gold staining of the blot, by revealing other protein spots in addition to the immunoreactive spots, has provided the necessary landmarks in turn facilitating subsequent alignment with the silver-stained gel using an appropriate 2-D analysis software program (Melanie 3).

This technique has identified two OB-R isoforms that differ in molecular mass by 2.2 kDa (60.2; 62.4) and differ in their isoelectric point by 0.2 pH units (5.78; 5.98). The level of macrophage contamination is determined by two macrophage markers, CD68 and cathepsin B. The studies include the determination of OB-R expression in a) unfractionated stromal cells vs. isolated pluri-differentiated mesenchymal progenitor cells; b) unstimulated cultures vs. cultures stimulated with a variety of cytokine/hormones including leptin itself. The studies also include mass spectrometric characterization

of the two OB-R isoforms detected by Western blotting in order to establish their exact structural differences.

#### Proteome Analysis of 2-D PAGE Separated Human BM Stromal Cell Membrane Proteins

BM stromal cells support the growth and development of normal blood cells as well as providing a haven for malignant leukemia/lymphoma cells. Focusing on stromal cell-surface proteins as potentially playing a role in cell-to-cell communication in normal as well as in abnormal hematopoiesis, the mixtures of stromal-cell plasma membrane, and plasma membrane-associated proteins were analyzed by a high-resolution, high-capacity 2-D gel electrophoresis. The 2-D system described utilizes an immobilized pH gradient gel (pH 4-7) in the first dimension and a mini nondenaturing but high-resolution lithium dodecyl sulfate-polyacrylamide gel electrophoresis (LDS-PAGE) in the second dimension. As identified by silver staining, this system has resolved greater than 800 protein spots in a pH interval of 2.5 units (4.25-6.75, the isoelectric pH range for most of plasma membrane proteins to migrate) and a molecular mass range of 10-150 kDa. Equally important, the system is compatible with high sample loads (up to 1.5-2.0 mg of total protein in up to 350- $\mu$ l sample volume). All the protein species identifiable by a silver stain that is compatible with subsequent mass spectrometric analysis have been analyzed by a 2-D gel software with respect to isoelectric point, molecular weight and mass abundance. The lectin-binding status of these proteins has also been determined by lectin blotting. Lectin blots and Western blots have subsequently been stained by a gold stain for detection of total proteins on the same PVDF membrane. Although gold-staining of the Western blot is not as sensitive as silver-staining of the gel, gold-staining of the Western blot generates the necessary landmarks for alignment with the silver stained gel, facilitating excision of spots of interest from the gel for identification by MALDI-MS. Representative protein spots were excised from gel and subjected to mass spectrometric profiling (MALDI-MS) and/or sequencing (Nano ESI MS/MS) with subsequent database searching, resulting in a productive identification of ten proteins. The protein digests are then submitted in a near-ready state for mass spectrometry. Upon receiving the MS data the group performs the database searching. MALDI/MS has been used, which identifies a protein on the basis of its characteristic mass sizes, as well as MS/MS studies that provide amino acid sequences of selected masses to identify proteins with enhanced specificity and confidence level. This work represents the first systematic attempt to analyze BM stromal cell proteins by high-resolution 2-D gel electrophoresis and provides the basis for a full-scale proteome mapping of the marrow stromal cells. The present work can facilitate the long-term goal of deciphering the hematopoietic support functions of BM stromal cells.

#### Modulation of Stromal Cell Plasma Membrane Protein Expression by TNF $\alpha$ /IL-4

The effects of TNF  $\alpha$ /TM on bone marrow stromal cell plasma membrane protein expression has been tested using the described system. TNF  $\alpha$  and IL-4 are regarded as negative regulators of hematopoiesis. Notably these cytokines, especially TNF  $\alpha$ , are elevated in marrow plasma of patients with myelodysplastic syndromes (MDS), the clinical hallmarks of which are anemia, leukopenia and thrombocytopenia (i.e., pancytopenia). TNF  $\alpha$  and IL-4 are thus possible mediators of hematopoietic dysregulation typical of MDS. TNF  $\alpha$ /IL-4 treatment of the stromal cultures induced dramatic changes in the protein profile. Initial studies using plasma membrane protein samples show reduced expression of at least 7 proteins and enhanced expression of 13 proteins.



Analyzing the Insulin-Like Growth Factor System in Human Marrow Stromal Cells by 2-D PAGE Analysis of BM Stromal Cell Culture Supernatants

Proliferation and development of normal and leukemic hematopoietic cells within bone marrow is regulated by interplay of various classes of molecules. These include cell adhesion molecules (CAMs), colony stimulating factors (CSFs), and cytokines as well as growth factors including insulin-like growth factors 1 and 2 (IGF 1 and IGF 2), which are small peptide homologs of prolinsulin. IGF 1 has known erythropoietic activity, whereas the function of IGF 2 is less clear. IGF 1 and 2 exert their activities through two types of receptors. The type I IGF receptor, a tyrosine kinase receptor highly homologous to the insulin receptor, binds to IGF 1 and IGF 2 with high affinity. The type II IGF receptor, a mannose 6-phosphate receptor that lacks intrinsic kinase activity, binds IGF 2 with high affinity and IGF I with low affinity. The type and number of receptors expressed on a target cell determine the strength of the IGF signal. One important key to understanding the IGFs' role in hematopoiesis is to appreciate how biological effects of receptors are modulated by larger soluble proteins, the IGF binding proteins (IGFBPs), which share no homology with the IGF receptors. Because IGFs and IGFBPs play important roles in cell growth and proliferation in many tissues, and because marrow stromal cells support hematopoietic growth and development, the patterns of expression of the IGF system components by marrow stromal cells cultured under serum-free conditions is necessary. To this end, unfractionated and purified stromal cells were analyzed, side-by-side, by Northern blotting, under varied stimulatory conditions for expression of IGFs and IGFBPs with surprising results. IGF 2 is constitutively expressed at a high level by macrophages in Dexter cultures; it is down regulated markedly by TNF  $\alpha$  alone; moderately by TNF  $\alpha$  plus IL-4; and unaffected by IL-4 alone. On the other hand, IGF 2 is minimally expressed by unstimulated MPCs, but is markedly up regulated by TNF  $\alpha$  alone or IL-4 alone; and moderately up regulated by combined TNF  $\alpha$  and IL-4. IGFBP4 is abundantly expressed both by macrophages and MPCs and is unaffected by cytokine treatment. In contrast, IGFBPs 5, 7, and 10, selectively expressed by MPCs, show no evidence of expression by macrophages and are unaffected by cytokine treatments. IGF 1 and the precursor to IOFBP 3 are not expressed in either macrophages or MPCs, either constitutively or after stimulation with TNF  $\alpha$ , IL-4 or both. In initial studies, bone marrow mononuclear cells expressed none of the IGFs or IGFBPs tested. These results provide important insights into the operation of the IGF system in stromal cells and it is likely that potentially novel IGFBPs can be uncovered by ligand blotting studies.

The present invention provides a large-format 2-D gel electrophoretic system for reproducible separation of MPG proteins and to prepare 2-D PAGE protein maps for normal bone marrow-derived MPCs (untreated and treated with representative cytokines, e.g. TNF  $\alpha$  or IL-4) and for MPCs derived from patients with representative pre-leukemic and leukemic conditions. The pre-leukemic conditions include—myelodysplastic syndromes (MDS) and the leukemic conditions include chronic myeloid leukemia (CML), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), and multiple myeloma (MM). The protein samples can consist of culture supernatants/secreted proteins; extracellular matrix (ECM) proteins; plasma membrane proteins solubilized using a three-step differential extraction protocol employing conditions of progressively increasing solubility; and whole cell lysate proteins similarly solubilized using the three-step differential

extraction protocol. This subproteome approach not only simplifies the 2-D PAGE electrophoretic protein patterns but also reveals additional proteins, which would otherwise have gone undetected.

Molecular Analysis Assay Involving the High-Resolution 2-D PAGE and Mass Spectrometric Identification of Gel-Separated Proteins

The completion of human genome project has provided a huge proteome database including the theoretical mass databases generated on the basis of site-specific cleavage employing proteolytic enzymes, such as trypsin and others. The availability of highly sensitive biological mass spectrometers together with the capability of bioinformatics to search extremely large amounts of data and identify the relevant proteins matching the mass spectrometry data provides the basis for the current excitement in proteomics. The focus of the interest is the BM MPC proteome as expressed under varied functional and disease states. The goal of the present invention is to identify BM MPC proteins that have possible functional and/or pathologic significance, that is, those proteins that show altered levels of expression in response to cytokine treatments and various leukemic states.

Until recently, the focus of the laboratory has centered on isolation and characterization of BM stromal cell adhesion molecules using a novel 2-D cell blotting technique. For this purpose, applicants have established an analytical 2-D mini gel system that separates stromal cell plasma membrane proteins using 18-cm long 4-7 pH range IPG strips in the 1<sup>st</sup> (Amersham Pharmacia Biotech). Subsequent to IEF, the IPG strip is cut into appropriately small pieces and subjected to 2 D separation using nondenaturing lithium dodecyl sulfate-polyacrylamide gel electrophoresis (LDSPAGE) and mini gels. The reason for using mini gels in the 2<sup>nd</sup> D is that they are compatible with a downstream functional assay involving cell adhesion. The stromal cell membrane proteins are blotted on to a PVDF membrane and assayed for hematopoietic cell-binding proteins directly on the blotting membrane. The system can be extended using 17-cm long 3-10 pH range IPG strips (Bio-Rad) for separation of stromal cell culture supernatants, ECM proteins, and whole cell lysates. As detected by silver staining of the gels, and analyzed by appropriate software (GelLab II or Melanie 3) this 2-D system has resolved greater than 800 membrane protein spots within a pH interval of 2.5 units (4.25-6.75) and a MW range of 10-150 kDa. Similarly, the ECM samples showed 475 spots; and conditioned media from BM stromal cell cultures grown under serum free conditions showed 524 spots. Not surprisingly, the total cell lysate of BM stromal cultures showed only 553 spots, most likely representing the abundant housekeeping proteins and masking detection of many functionally relevant proteins. These observations provide the rationale for the proposed subproteome approach involving the use of differential solubilization of sample proteins and multiple large gels. Membrane proteins thus far identified by mass spectrometry followed by database searching; proteins are identified by standard Western blotting. Select IGF binding proteins were identified by ligand Western blotting. The blotting shows the identification of IGF-binding proteins (IGFBPs) using 2-D ligand blotting. The conditioned media from BM stromal cultures grown under serum-free conditions were concentrated using Microcon concentrator, and proteins were fractionated using a high-resolution 2-D PAGE. The separated proteins were electroblotted onto PVDF membrane and subjected to Western ligand-blotting assays using 1-125 labeled IGF-2, resulting in the identification of a series of



IGFBPs (up to 30 spots). Notably, TNF  $\alpha$  treatment of the cultures down-regulated two LGFBPs and up-regulated IGFBP labeled 6.

By necessity the protein work began on BM stromal cells using a nondenaturing (LDS-PAGE) mini gel system that contained no reducing/alkylating agents. To preserve the function of 2-D gel separated proteins many otherwise powerful sample preparation methodologies designed for proteomic studies (such as multiple surfactant solution, MSS) were avoided. While mini gels are convenient and allow comparison and information transfer to large-format gels, they are less sensitive.

#### Subproteomes According to Sample Prefractionation

In order to be able to identify the low-abundance proteins implicated in the regulatory and pathologic processes, a number of approaches to prefractionation of the whole cell lysates have been described. Applicant studied the subproteomes of secreted proteins from BM stromal cell culture supernatants as well as ECM proteins. Notably, the ECM protein samples can be a rich source of functionally relevant cytokines and chemokines since the latter are known to mediate function by binding to ECM. In addition, the plasma membrane and whole cell samples were subjected to the 3-step sequential solubilization protocol shown. The solubilizing solutions can be prepared in-house or purchased commercially (Bio-Rad). The first step involves the use of Tris base, which can solubilize the peripheral membrane proteins and cytosolic proteins. These proteins are lyophilized and subsequently solubilized prior to 2-D PAGE in a standard solubilizing medium (the modified O'Farrell cell lysis solution containing urea, CHAPS, DII, Iris, ampholytes and appropriate protease inhibitors). The resulting pellet can also be solubilized in the standard 2-D solubilizing medium and subjected to 2-D PAGE. Because the standard solution cannot solubilize some proteins, the membrane-rich pellet is finally solubilized in a potent multiple surfactant solution (MSS) consisting of urea, thiothrea, C1-LAPS, zwittergent 3-10 and tributyl phosphine (TBP) in addition to Iris base and ampholytes that is compatible with subsequent IEF. The MSS has been shown to solubilize the hydrophobic proteins with as many as 12 transmembrane regions (TMRs), facilitating their 2-D analysis. Another final step incorporating 1% SDS in boiling sample buffer can be used to test by 1-D SDS-PAGE if any proteins remained unsolubilized after these extractions (notably, SDS extract is unsuitable for 2-D PAGE analysis since SDS interferes with IEF). The prefractionation step clearly reduces the complexity of the sample. Thus, the serial extractions not only simplify the gel images and reduce spot overlapping frequently encountered in single-step extractions but also correlate closely with the cellular location of specific proteins, providing clues to their function. The prefractionation strategy can be extended to enriching low-abundance proteins in culture supernatants by selective removal of contaminating albumin using an Albumin Depletion Kit (containing Cibachron Blue resins) (Genomic Solutions, mc). Similarly, membrane glycoproteins can be enriched by a Glycoprotein Enrichment Kit (containing lectins) prior to 2-D PAGE analysis (Genomic Solutions, Inc.).

#### Subproteomes According to Overlapping pH Gradients

Using a series of medium-range and partially overlapping pH gradients (3-6, 5-8, 7-10, each 17-cm long) (Bio-Rad) can enhance reproducibility and resolution by creating "virtual" gels with up to 40 cm equivalent of PI separation across a pH 3-10 range. Alternatively, a combination of two pH gradients, pH 4-7 and pH 6-11, each 24 cm-long (Amersham Pharmacia Biotech) can be used, also providing a "virtual" separation distance of 40 cm across a pH range of 4-11. These strips

accept micropreparative sample loads (1-2 mg). Notably, a given sample of cells yields a total of 8 protein samples. These samples include one protein sample composed of conditioned medium, one protein sample comprising of ECM proteins, three protein samples derived from plasma membrane lysates and three protein samples derived from total cell lysates, following application of a three-step protein extraction protocol to purified plasma membranes and total cells. Eight protein samples can thus translate into 24 large format (18 cm) gels corresponding to three overlapping 1st D gels; or 16 extra large format gels (24 cm) corresponding to two overlapping 1st D gels. Proteomics is no longer considered a single 2-D gel study. Taking advantage of the common spots in the 2nd D corresponding to overlapping regions, PDQUEST software can allow "stitching together" of the constituent gels, creating the so-called "cyber gel" providing a composite map for each protein sample. The data generated can be stored in an internet-accessible 2-D PAGE database in the form of 8 submaps. Three of these submaps correspond to plasma membrane proteins representing 3-step solubilization; one of them corresponds to secreted proteins; one of them corresponds to ECM proteins; and 3 of them correspond to total cell lysate proteins representing 3-step solubilization. These submaps can be linked to a master synthetic gel, a conglomerate of the submaps, representing the so-called "cyberproteome" of MPCs. Given the ability to run up to 12 IPG strips per 1st D gel (using IPGPhor) and 10 to 12 large or extra large SDS-PAGE gels per 2<sup>nd</sup> D gel run (using Hoefer DALI and Ettan DALI II, respectively), the resulting number of gels can be well within the manageable workload of one person (36). Although not easily accessible now, some innovative technological developments are on the horizon, e.g., development of fluorescence 2-D difference gel electrophoresis (DIGE), which could minimize the tedium. Unlike the current practice of running different protein samples on separate gels, and then staining and comparing the gels, DIGE technology uses matched, spectrally resolvable dyes (e.g., Cy2, Cy3 and Cy5) to label protein samples prior to 2-D separation. Differentially labeled protein samples are mixed and co-separated by 2-D electrophoresis, allowing analysis of at least three samples on a single gel. Gels are scanned and proteins are subjected to image analysis using appropriate software. Alternatively, one can use a highly sensitive silver stain to visualize the proteins after electrophoretic separation. Notably, the silver stain is compatible with subsequent mass spectrometry analysis.

#### Follow-Up Strategy for 2-D PAGE Using the So-Called Ultrazoom LPG Gels with Narrow-Range pH Gradients

Commercially available narrow-range IPG strips include pH 3.5-4.5; 4.0-5.0; 4.5-5.5; 5.0-6.0; 5.5-6.7. These are available as 18 cm and 24 cm-long strips, consequently allowing spanning of 1 pH unit over a distance of 18-24 cm and providing extraordinary resolution. By using narrow pH gradients (IpH unit) up to 10 mg of protein would be loaded onto a single IPG gel strip, either by repeated sample cup application or by in-gel rehydration without incurring vertical or horizontal streaking. Employing a combination of such narrow-range overlapping IPG strips, one study utilized up to 40 2-D gels for analysis of a single protein sample. The preference is not to follow such extreme approach but rather to use these gradients as a backup in situations where a functionally relevant protein is first detected by the front-end strategy but could not be studied by mass spectrometry for lack of adequate resolution or due to overlapping spots. The 24-cm long narrow IPG strips can be subjected to 2<sup>nd</sup> D using correspondingly extra large slab gels (the required precast, plastic-backed gels can be purchased from Amersham Pharmacia Biotech). However,

the "giant 2-DE" 30×40 cm size gels are impractical to handle. The situations for the use of narrow range pH gradients include situations like detection of proteins by Western blotting using anti-phosphotyrosine antibodies or Western ligand blotting using labeled IGF 1 or 2, which are probably more sensitive than silver staining. Consequently, these assays identify the functionally relevant proteins but without providing the actual identity of the individual proteins. Because the front-end strategy can at least provide the range of the phosphoprotein or the IGF-binding protein identified, on the basis of this information samples can be subjected 2-D PAGE using the appropriate ultrazoom IPG strip, which as indicated above can permit loading of several mg of protein sample. Extra large precast slab gels (26×20 cm) with plastic backing suitable for running the 24-cm long ultrazoom IPG strips and the appropriate electrophoretic system (Ettan DALLI 2 dimension electrophoresis system) that runs up to 12 of these gels are commercially available (Amersham Pharmacia Biotech).

#### Summary of Subproteome Strategy

The subproteome approach involves: 1) Cellular fractionation involving isolation of purified MPCs. 2) Subcellular fractionation involving preparation of functionally relevant protein sets. These include: 2a) secreted proteins such as colony stimulating factors (CSFs), cytokines, etc in the conditioned media; 2b) ECM proteins such as cell adhesion molecules (CAMs), etc; 2c) plasma membrane proteins such as various receptor molecules, CAMs and components of cell signaling systems, etc; 2d) finally, whole cell lysate proteins that include some of these proteins plus cytosolic and nuclear proteins. The cytosolic and nuclear proteins can be a rich source of target proteins for phosphorylation with a regulatory function.

#### 2-D PAGE Data Capture and Analysis

2-D PAGE data capture and analysis can be performed using standard equipment and protocols. Silver-stained gels can be scanned using an imaging densitometer and processed with QuantOne software (Bio-Rad) whereas gels stained with fluorescent Sypro Ruby (with 450 nm in the excitation range) can be scanned using a STORM 860 gel and blot imaging system and processed with ImageQuant Solutions software (Amersham Pharmacia Biotech). A number of factors, including differences in sample preparation and loading, staining and image acquisition can influence the reproducibility of 2-D gel protein separation. Quantitative data are reported as spot volumes (integrated spot densities). In experiments comparing replicate 2-DE patterns of the same sample or 2-DE patterns of samples from different individuals, the spot volumes in each pattern are scaled to correct for differences in the total amount of protein loaded onto each gel. These variations are compensated by accurately comparing the quantity of any spot across multiple gels. These operations can be performed using a dedicated 2-D gel analysis software, Melanie 3. This program can analyze such variations by scatter analysis and can compensate for varying staining absorption across proteins by normalizing protein expression change. Varying stain intensities and sample sizes can be compensated for by relative spots quantification. The Melanie 3 software also has the capability to merge several gel electrophoretic patterns from the same sample into a composite gel, providing fine control over the included proteins. Finally, the software can compensate for gel distortions caused by variations in protein migration through alignment of the gels.

#### Mass Spectrometric Instrumentation

Examples of such mass spectrometers include, but are not limited to, Voyager DE Pro (Applied Biosystems, formerly

Perceptive Biosystems, Inc.) and QSTAR (Applied Biosystems). Voyager DE Pro is a matrix-assisted laser desorption time-of-flight mass spectrometer (MALDI-TOF) that can be operated in a linear mode for the analysis of large biomolecules or in a reflector mode for high-resolution analysis of smaller molecules, i.e., peptides. The MALDI-TOF instrument also utilizes delayed extraction technology that results in greatly increased resolution, sensitivity and mass accuracy. This is the instrument of choice for high throughput analysis, with a capacity of up to 100 samples per sample plate. On the other hand, the QSTAR is a hybrid quadrupole-quadrupole-time-of-flight mass spectrometer. Samples are introduced in solution and are ionized by electrospray. For samples requiring the highest sensitivity, Dr. Jackson utilizes a low flow rate (25 nl min<sup>-1</sup>) electrospray called nanoelectrospray, typically requiring only 1-2 µl of a solution for sample analysis. The QSTAR instrument yields data quite similar to those obtained from the MALDI-TOF instrument, except that the QSTAR data analysis is somewhat more complex due to the multiple charging of peptides by the electrospray process compared to the single charging applied to peptides on the MALDI-TOF instrument. One important additional characteristic of the QSTAR is its ability to determine structural information from sample molecules by tandem MS/MS. This is achieved by effectively "purifying" selected molecules within the mass spectrometer's first quadrupole section. For analysis of peptides produced by tryptic digestion, a single MS experiment is initially performed to determine the masses of components present in the mixture. Next, MS/MS experiments are carried out to select specific peptides for de novo amino acid sequence determination. Typically 2 µl of peptide mixture is sufficient for determining the sequences of ten to twelve peptides.

#### Mass Spectrometric Analysis by MALDI

The scheme for mass spectrometric analysis of in-gel tryptic digests of proteins for the purpose of protein identification consists of several steps. First, the peptides extracted from the gel must be cleaned and concentrated. The cleanup is necessary to remove residual detergent and other non-peptide materials that can interfere with the analysis of the tryptic peptides. This step involves binding of the peptides to a Microcon-SCX adsorptive microconcentrator. This is a cation exchange membrane held within a microcentrifuge device. At low pH, the peptides bind to the negatively charged membrane, while uncharged or negatively charged molecules pass through. After a brief wash, the peptides are eluted from the membrane in two 25-µl steps of 1.5 N ammonium hydroxide in 1:1 methanol/water. The samples are then speed-vac dried for 10 minutes, and fresh solvent is added for additional treatment to concentrate the sample prior to MS analysis. Initially, all samples can be analyzed by MALDI-TOF MS. For this analysis, the sample from the Microcon-SCX elution can be dissolved in 0.1% trifluoroacetic acid (TFA) in water and loaded on a ZipTipC 18 Pipette tip. The tip is then washed with the same solution and the peptides are then eluted directly onto the MALDI-TOF sample plate with 2 µl matrix solution (cyano-4-hydroxy-cinnamic acid, 10 mg/ml in 0.1% TFA in 1:1 acetonitrile/water). The spotted sample is dried at room temperature for at least five minutes before the sample plate is loaded in the instrument. The instrument calibration is performed externally by the addition of a calibration mixture to the sample plate. Samples are calibrated internally if the known tryptic autodigestion peptides are observed in the sample. This can be used as long as the specific type of trypsin used in the proteolytic digestion step is known. After data collection, the data can be further processed in two ways. First, the data can be treated by noise reduction software and

second, it can be deisotoped. Software for both operations of these programs are standard features of the Data Explorer system provided with the Voyager DE Pro mass spectrometer. The obtained peptide mass data can be subjected to peptide fingerprint analysis utilizing one of the protein database search sites on the Internet, such as Mascot or MS-Fit. While each of these search sites has access to several databases, one can initially select either OWL or NCB1nr. One can search the database with a standard set of criteria without using a species filter. The practice is to select three variable modifications to allow for conversion of peptide N-terminal glutamine to pyroglutamate, and oxidation of methionine residues; allowing for up to one missed cleavage. Neither the protein MW nor the PI can be used as a search parameter (these, however, can be used for subsequent validation of the matched protein). Also important is that expected peptide masses of known potential "contaminants" such as keratin and trypsin can be excluded from analysis. Finally, the peptide mass tolerance can be set to  $\pm 0.15$  Da relative to the monoisotopic MW of the singly charged peptide ion. Positive database hits are scored with a MOWSE number. The higher the number of hits the greater the confidence level. The database search algorithm relates the significance level for a given search. If a high MOWSE score is obtained indicating an unambiguous match, one can consider the protein positively identified, otherwise the sample can be subjected to analysis by use of the QSTAR mass spectrometer.

#### Mass Spectrometric Analysis by Nano ESI MS/MS

Samples that require analysis utilizing the QSTAR, following cleanup by Microcon-SCX adsorptive microconcentrator, can be concentrated by binding the peptide mix to a small amount of POROS R12 reversed-phase C18 chromatographic support packed into a nanopurification capillary. The packed capillary column volume is  $\sim 10$ - $15$  nl. The sample, dissolved in  $10$ - $p.1$  of 5% acetic acid in water, is applied to the capillary by use of a ten- $\mu$ l gel loading pipette tip. A brief centrifugation forces the liquid down the capillary so that the peptides can bind to the support. The support is then washed with  $10$ - $15$  p.1 of 0.5% acetic acid in 1:50 methanol/water. The peptides are eluted from the purification capillary into a nanospray capillary by the addition of 2  $\mu$ l of 0.5% act id in 1:1 methanol/water followed by brief centrifugation with the nanospray capillary stacked just below the purification capillary in a micropurification holder (MDS Protana). Initially, data for a single MS run is collected. The peak masses are labeled and peptides are selected for potential MS/MS sequencing by locating those that appear to be doubly charged. Most peptides resulting from a tryptic digest can have a significant doubly charged form, which is ideal for MS/MS sequencing. The first quadrupole of the QSTAR is tuned to pass a 2 Dalton window for the pre-selected doubly charged peptide ions, one at a time, for fragmentation by collision with low-pressure argon gas in the second quadrupole. Collision energy is adjusted for each peptide to obtain the best possible MS/MS spectra. Data are collected long enough to get good quality spectra. After MS/MS spectra are collected for all selected peptides, the data are manually interpreted. Internet protein database searches are performed similar to that for MALDI-TOF peptide fingerprint, except that the search is a partial amino acid sequence search with mass information (i.e., Mascot, employing Sequence Query format). The search criteria cannot screen for a species or a protein MW or PI (which, however, can be used for subsequent validation of the protein matched). Also important is that expected peptide masses of known potential "contaminants" such as keratin and trypsin can be excluded from MS/MS analysis. One missed cleavage can be allowed and two variable modifications can be

selected, carbamidomethylation of cysteine and oxidation of methionine. The tolerance of the peptide monoisotopic mass can be set to  $\pm 0.3$  and the MS/MS tolerance can be set to  $\pm 0.2$ . This type of search generally requires only two or three peptide sequences consisting of three of the amino acids per peptide to obtain a statistically significant match (a high MOWSE score). Once a match is identified, a list of the matched peptide's theoretical MS/MS fragments can be generated to compare with the observed fragments to further confirm the correctness of the match.

#### Establishment of Large-Format 2-D PAGE Protein Maps for MPCs Derived from Normal BM

The normal cell samples include, A) Untreated normal MPCs; B) Normal MPCs treated with TNF  $\alpha$ ; C) Normal MPCs treated with TNF- and IL-4. Each cell sample can generate a total of 8 protein samples, 1) culture supernatants/secreted proteins (1 protein sample); 2) extracellular matrix (ECM) proteins (1 protein sample); 3) plasma membrane proteins solubilized using a three-step differential extraction protocol employing conditions of progressively increasing solubility (3 protein samples); 4) whole cell lysate proteins similarly solubilized using a 3-step differential extraction protocol (3 protein samples). Each protein sample can generate 3 large format 2 D gels (corresponding to 3 medium-range, overlapping IPG gradient gels, pH 3-6; 5-8; 7-10). This means each cell sample can generate 24 large format 2 D gels, leading to generation of at least 72 large format gels for analysis of normal MPCs. To account for duplicate or triplicate samples, the gel number falls in the range of 200-300.

#### Establishment of Large-Format 2-D PAGE Protein Maps for MPCs Derived from BM Involved with Representative Pre-Leukemic and Leukemic Conditions

The disease-associated MPCs include those from MDS, CML, AML, CLL, ALL, and MM. As above, each MPC sample can generate 24 large-format 2 D gels. With 6 such diseases being studied, the gel number can reach 144. To account for duplicate or triplicate samples, the gel number falls within the range of 400-500. The use of IPGPhor, together with ready-made IPG strips, permits sample in-gel re-hydration and performance of unattended IEF overnight by adding automation to the 2-D procedure.

Using High-Resolution Proteomics and with the Added Power of High-Throughput Robotics, Identify on a Larger (Semi-Comprehensive) Scale the MPC Proteins that are Differentially Expressed in Conditions that Simulate Pre-Leukemic Bone Marrow (Following Stimulation with Different Cytokines); and in Actual Pre-Leukemic Disorders (MDS) as well as in Overt Leukemias (CML, AML, CLL, ALL, MM)

A robotically guided system facilitates excision of protein spots (by a spot cutter or picker) from 2-D PAGE gels, transfer of protein samples to 96-well microplates, and automated protein digestion in the microwells. Such a system reduces the time and labor relative to manual procedures and provides high throughput while minimizing keratin contamination from human skin, a frequent problem in proteomics research. The preferred method is to excise all spots from a gel but to process only the spots of interest, storing the remaining excised proteins frozen at  $-70^{\circ}$  C. for a later use. The robotic components can include MALDI slide spotter in addition to an automated protein spot picker and digestion station.

#### Example 8

##### Methods

The present study involved microarray analysis of 23 samples and a corresponding number of chips. The samples were obtained from 4 normal healthy adult human subjects,

consisting of mixtures of unfractionated stromal cells (collective USCs or cUSCs, 8 samples), Percoll gradient-purified MPCs (collective MPCs or cMPCs, 5 samples) and single-cell MPCs (sMPCs, 10 samples) obtained by laser-capture microdissection (LCM). The study design allowed for adequate controls and replicates appropriate for a comprehensive gene expression profiling of normal BM stromal cells. The isolated single stromal cells were selected on the basis of morphology. Wright-Giemsa stained cytospin preparation revealed characteristically large cells with a relatively irregular nucleus and cytoplasm compartmentalized into ectoplasm and endoplasm. Subsequently, applicant identified a hematoxylin stain as a substitute for Wright-Giemsa stain. The hematoxylin stain is simpler to use and provides morphologic detail sufficient to allow recognition and isolation of these cells by laser capture microdissection and does not interfere with the downstream microarray testing (see details under Materials & Methods). The photomicrographs of 10 stromal cells that have been subjected to microarray testing are shown in FIG. 15. To serve as controls and facilitate comparison, applicant analyzed side-by-side 8 samples of unfractionated stromal cells that are "contaminated" by up to 35% macrophages and 5% hematopoietic cells (referred to collective USC, or cUSC), and 5 samples of Percoll-gradient purified stromal cells, up to 95% pure (referred to collective MPC, or cMPC to distinguish from sMPC). RNA isolated from sMPC samples was subjected to 2 rounds of amplification using RiboAmp kit (Arcturus, Inc) prior to in vitro transcription (IVT). In contrast, RNA samples isolated from cUSCs and cMPCs were used without amplification for IVT. The subsequent steps of microarray testing were standard for all 3 types of samples and are schematized as follows: Preparation of total RNA → generation of cDNA → preparation of ds cDNA → in vitro transcription into cRNA → fragmentation of cRNA → hybridization of target RNA to a microarray of known genes (Affymetrix U95Av2 oligonucleotide microarray, with 12,625 probe sets) → Signal quantification and first-tier analysis using the microarray quantification software, Microarray Suite (MAS v. 5, Affymetrix, Inc). The presence of a gene within a given a sample was determined at a detection p-value of <0.05, according to the statistical expression analysis algorithm employed by MAS v.5, and was graded absent (A), marginal (M) or present/positive (P).

#### Assessment of RNA Amplification Method

Since single-cell microarrays are relatively novel, applicant critically reviewed the data with respect to two important statistics that would reflect on the reliability of RNA amplification assay procedure employed before analysis involving data-mining techniques. a) 3': 5' ratios of housekeeping control genes: As shown in Table 9, these ratios were close to 1 in the standard unamplified samples, whereas, they were increased in the amplified samples. Although this is to be expected due to preferential amplification toward 3' end, since amplification may not proceed all the way up to 5' end, applicant wanted to exclude possible sample degradation. For reasons unclear, in the case of ACTB (beta actin), the 3': 5' ratios were highly variable across single cell MPG samples. In any event, the 3': 5' ratios in case of GAPD (glyceraldehyde 3-phosphate dehydrogenase) were relatively tight, suggesting no evidence of sample degradation. Furthermore, both GAPD and ACTB gene probes that were employed as part of the standard gene probe set yielded relatively stable signals across replicates in each sample type, which is further evidence of intactness of RNA samples targeted for microarray analysis. b) Number of genes present or detected: As outlined in Table 9, the amplified single cell MPG RNA samples expectedly showed significantly lower number of genes com-

pared to the standard RNA samples (on average 34% vs. 46% of the genes etched on the array). The fact that the number is relatively constant across single cell replicate samples is further indication of the reliability of the data. Notwithstanding the shortcomings of the amplification procedure, it is important to bear in mind that the conclusions are based only on those stromal cell genes that are detected commonly across unamplified cMPCs and cUSCs as well as in amplified sMPCs (but do not include the genes undetected or the genes selectively detected in sMPCs).

#### Data Mining and Reproducibility of Overall Procedures

The thrust of the present invention is to identify genes that are relatively uniformly expressed across normal untreated bone marrow stromal cell samples, regardless whether they are of single cell type or collective cell samples, purified or unpurified. As detailed under Materials & Methods, GENE-SPRING was used to achieve the following data-analysis objectives: a) Filtering for genes reliably detected in each sample group by eliminating the genes with weak expressions that are statistically close to the background estimate. b) Filtering for genes that are active or "present" across replicates in each sample group. c) Exclusion of genes with weak expressions from genes "present" in each sample group. d) Preparation of master stromal cell gene list by intersecting gene lists from step (c) (as shown in FIG. 16). These steps have led to identification of a list of 2755 genes that are detected in at least 7 of 8 cUSC samples AND 4 of 5 cMPC samples AND 9 of 10 sMPC samples, i.e., in at least 20 of 23 stromal cell samples investigated. The main conclusions of the present report are based on this "stromal cell gene list" that is broadly representative of all 3 types of stromal cell samples investigated, and not on a gene list that is selective to sMPCs. A hallmark of the quality of microarray data can be discerned from hierarchical cluster analysis of replicates, which involves the principles of vector algebra. An array of numbers representing expression levels of a particular gene in terms of normalized signal intensity in a group of samples is considered a gene expression vector. Likewise, an array of numbers showing expression levels of a group of genes by a particular tissue sample is considered a tissue expression vector. In the case, applicants have 2755 gene expression vectors and 23 tissue or sample expression vectors. These vectors are amenable to algebraic treatment, facilitating calculation of similarity between any two gene- or tissue-expression vectors on the basis of a correlative metric or a similarity-measure employed, e.g., Euclidean angle. Grouping together of two samples on the basis of these principles signifies that they are most closely related out of all the samples in terms of their overall pattern of gene expression. Construction of a bone marrow stromal cell hierarchical tree has enabled visualization of global gene expression patterns across replicates and conditions. As shown in FIG. 17, stromal cell genes that are expressed at a relatively lower level in amplified samples (sMPCs) are clustered to the left of gene tree, genes that are more strongly expressed in sMPCs are prominently figured in the middle of gene tree, and genes that are expressed approximately at same level as in unamplified samples (cMPCs and cUSCs) are clustered to right of gene tree. Even more important as noticeable on the sample or experiment tree, hierarchical clustering segregated the members of each sample type into a separate group (cMPC, cUSC and sMPC). Note within each sample type, corresponding subject replicates clustered together with minor exceptions. This is a reflection on the reproducibility of the overall assay-procedures employed, which encompass a variety of stages and steps in addition to target RNA amplification prior to in vitro transcription (see Materials & Methods for details).

Finally, it is important to keep in mind that the measured level of a transcript following amplification does not necessarily provide a quantitative estimate of gene expression, but only provides a qualitative indication that the gene is transcriptionally active, which by itself is sufficient grounds for the conclusions arrived at in the present report. As shown in FIG. 18, the expression of genes within the stromal cell gene list ranges from 0.2 to 6 (on the log scale) in unamplified samples (cMPC and cUSC) and from 0.02 to 36 in amplified samples (sMPC), thus showing much greater variability in the amplified samples. For illustration purpose, the coloring of gene expression curves (following the linear color bar shown on the right) was based on the gene expression pattern of a particular single-cell sample, SCA1. Note that the genes that are detected at a low level in this sample (as indicated in blue) are not necessarily expressed at a low level in unamplified samples (as read by the log scale on Y-axis). In fact, a significant number of them are expressed at a high level in the unamplified samples. This finding together with the observation that amplified samples detected about 34% of genes as opposed to unamplified samples detecting about 46% of genes tested (Table 9), strikes a cautious note that some genes do not amplify at all by the method used, and other genes amplify to a sufficient degree to be detectable (shown in blue), while some other genes amplify to a degree equal to (in yellow) or surpassing (in red) the amounts in the collective samples. (The curve shown in white is the housekeeping gene, GAPD.) The statistical algorithm as implemented in the latest version of Microarray Analysis Suite (MAS v.5) determined that a gene within a given sample as positive, regardless of grading. To overcome the limitations of the amplification procedure employed, applicant focused only on genes that were positive in at least 20 of the 23 stromal cell samples investigated.

A stromal cell gene list is expected to be representative of typical stromal cell gene expression profile. Such master gene list forms the basis for derivation of all other stromal cell gene lists, organized in accordance with lineage or functional categories. As depicted in FIGS. 19A-19F, and listed in Tables 10 through Table 17, that were prepared according to lineage/functional assignment, the findings show that isolated single cells simultaneously express genes associated with diverse mesenchymal cell lineages, namely osteoblasts, muscle cells, fibroblasts, adipocytes, epithelial cells, endothelial cells, nerve cells and glial cells, providing confirmation of the existence of a pluridifferentiated progenitor cell type. By definition the stromal cell genes are present in at least 4 of 5 collective MPC samples AND 7 of 8 collective USC samples AND 9 of 10 single cell MPC samples; consequently, they are active in at least 20 of 23 samples tested, representing a typical genomic profile of stromal cells. The following gene lists are sub-lists of the master stromal cell gene list consisting of 2,755 genes. The stromal cell gene list contains a number of genes that are capable of causing endothelial differentiation and vasculogenesis within the marrow microenvironment; however, these genes themselves are not necessarily endothelial cell markers. In fact, stromal cells express a gene, EDF1, the expression of which inversely correlates with endothelial cell differentiation within the stromal cells. Of the 67AFFX control genes present on the U95A v2 chip, 22 genes are detected in at least 7 of 8 cUSC samples, 24 genes are detected in 4 of 5 cMPC samples and 19 genes are detected in at least 9 of 10 sMPC samples. Thirteen of these genes are present in the stromal cell gene list, i.e., in 20 of the 23 samples investigated.

As evident from these gene lists, note that an isolated single stromal cell simultaneously expresses transcripts for epithe-

lial and neuroectodermal cell types as well. Departing even further from the initial thinking, the findings add to the evidence that the MPCs within the Dexter system might represent a form or stage of the progenitor cell that is common to nonhematopoietic and hematopoietic cells. As depicted in FIGS. 20A-20F, and listed in Table 18 through Table 21, the isolated single stromal cells express transcripts that are typical of hematopoietic cells, in particular precursor B cells. That BM stromal cells express CD10 (CALLA) is not novel since BM stromal cells as well as endometrial stromal cells and normal breast myoepithelial cells are known to express CD10. However, the expression of CD19, CD79A and immunoglobulin enhancer binding factors E12/E47 (proto-oncogene TCF3) by BM stromal cells is unforeseen, and forms the basis for postulating the existence of a common progenitor with B cell lineage. B-cell progenitors typically display the phenotype, (CD45±, CD34±, CD20±, (CD10+, CD19+, CD79A+, HLA-Dr+), which as reported here is also displayed by isolated single stromal cells at least at the transcriptome level.

CD45 positivity by cMPC and cUSC samples is attributable to coexisting or contaminating hematopoietic cells in these samples as evidenced by concurrent positivity for myelomonocytic markers CD13, CD33 and CD14 (Table 21). However, a products or transcripts for CD45 and CD19 are most likely present in stromal cells at a basal level that is beyond the detection limits of conventional techniques, e.g., immunocytochemistry and Northern blotting, respectively. Conceivably, two rounds of amplification prior to IVT sufficiently increased their transcript levels to be detected by microarray analysis. In fact, the CD45 levels were several-fold lower in cMPC and cUSC compared to CD45 levels in sMPC, and CD19 was undetectable in unamplified samples. Finally, note that CD45 and CD19 are not isolated examples in this regard since applicant has identified at least 200 other genes that are uniquely present across sMPC samples but undetectable in cMPC and cUSC (FIG. 16).

#### The Issue of Stromal Cell—B Cell Connection

Although no analog of Bursa of Fabricius exists in mammals, bone marrow is generally regarded as the site of B-cell generation. While the Dexter type stromal cell culture system was devised for investigation of hematopoiesis, specifically myelopoiesis (see Introduction), Whitlock and Witte developed another system for the study of B-lymphopoiesis. Whitlock-Witte cultures, like Friedenstein cultures, are grown in the absence of hydrocortisone and horse serum. When stromal cell layers in W-W cultures are seeded with fresh bone marrow as a source of B cell precursors or with purified B cell precursors, the latter then differentiate into mature B cells. On the other hand, although Dexter cultures do not promote B cell maturation, Dexter cultures do contain B cell precursors, which upon switching of culture conditions from those of Dexter to Whitlock-Witte, differentiate into mature B cells. While BM stromal cells in one form or another are definitely known to support B-lymphopoiesis, it has not been so clear as to whether stromal cells actually give rise to B-cell precursors. Evidence for a progenitor cell common to stromal cells and hematopoietic cells has been coming to light piecemeal in the form of isolated reports. 1) Singer J W et al. in 1984 in the course of investigating bone marrow cultures from human patients with clonal myeloproliferative disorders showed that the nonhematopoietic stromal cells were derived from the same clonal progenitors that were involved by the hematopoietic neoplasm, as revealed by G6PD marker analysis. 2) Huss R et al. in 1995 in the course of investigating a canine BM stromal cell line showed that the adherent stromal cells had "turned" into nonadherent hematopoietic cells, espe-

cially when the latter were cultured in presence of stem cell factor. 3) Pessina et al in 1997 showed that a particular murine stromal cell line upon stimulation with bFGF, expressed a B-cell phenotype, including CD45R and surface immunoglobulin. Although not by design, applicant shows for the first time that isolated single stromal cells express transcripts that are typically associated with hematopoietic lineage, namely, CD45 and CD19, as well as relevant proto-oncogenes and transcription factors. These results are strongly supportive of the existence of a progenitor cell common to bone marrow stromal cells and hematopoietic cells, particularly the bone marrow-derived (B) lymphocytes. Note that the study involves no feeder cells, no embryonic stem cells, no cell lines and no colonies of cells. Contrasting with the existing literature, the present study embarks on a new path of investigation entailing gene expression analysis of single, primary, normal human stromal cells that suggest a broad capacity for multilineage differentiation. On this model, progenitor cells express genes that are characteristic of any of the lineage fates that these cells are capable of adopting.

#### Perspective on Pluripotentiality vs. Pluridifferentiation

The present investigation involves isolated single stromal cells, consisting of 10 cells from 4 different individuals (FIG. 15). The cell culture system has been earlier characterized at light microscopic level, ultrastructural level and by karyotypic analysis, showing no evidence to suggest the artifacts discussed. Spontaneous cell fusion most likely involves monocytes/macrophages, forming multinucleated giant cells; however applicant observed no expression of myelomonocytic marker genes by isolated single stromal cells (Table 12). Keep in mind that the readout of *in vivo* transdifferentiation studies involves localization of different lineage cells in different tissues or organs; such a situation only requires fusion between two cell types (one donor cell and one recipient cell) for the investigators to believe the artifact as transdifferentiation. In contrast, applicant shows here presence of genes for a variety of cell-lineages simultaneously within the same cell. The probability of an array of different cell types fusing into one cell which then masquerading as a pluridifferentiated cell, and that too happening with 10 of 10 cells investigated, is in the opinion close to zero. There has never before been an opportunity to assess the extent of differentiation within these multipotential progenitor cells in molecular terms at the single-cell level. Theoretically, a) A stem cell can directly become a terminally differentiated cell, or b) A stem cell can enter a phase of multilineage differentiation prior to becoming a single-lineage, mature cell. To the knowledge, this study is the first systematic attempt to answer these questions at the single cell level by using the marrow stromal cells as a model. Although numerous forward-looking reviews exist on the topic of single-cell genomics, only a rare report is available on the actual application of this technology. Applicant has applied this frontier technology to show that a phase of multilineage differentiation indeed exists at least in Dexter-type stromal cells. Pluripotentiality of the bone marrow mesenchymal stromal cells in terms of their ability to become muscle cells, bone cells, fat cells and fibroblasts under select culture conditions has been described by other investigators. Instead of documenting another example of the phenomenon *per se*, the results provide an independent validation of the studies on transdifferentiation by casting light at the molecular basis of cellular plasticity. Finally, to borrow a concept from the clinical practice setting, a morphologically "poorly" differentiated neoplasm expressing hematopoietic markers is classified as a leukemia/lymphoma and treated as such. Similarly, a morphologically "undifferentiated" neoplasm marking for epithelial gene expression is diagnosed as a carcinoma

and treated according to the protocols designed for a carcinoma. It is in this sense that applicant uses the term "pluridifferentiated" as opposed to "pluripotential" to characterize the BM stromal cells. Notwithstanding the semantics, applicant shows that the pluripotent stromal cells are pluridifferentiated, at least at the molecular level.

#### Conclusions

The findings of the present study clarify the on-going controversy as to the co-existence of multiple stromal cell types vs. one stromal cell type with co-expression of multiple phenotypes within the Dexter system of BM stromal cell cultures. An isolated single stromal cell from these cultures simultaneously expresses an array of phenotypes, i.e., osteoblasts, fibroblasts, muscle cells, adipocytes, epithelial cells, endothelial cells, neural cells/glia cells and even hematopoietic cells, in particular, B-lymphoid progenitors, thus documenting its wide differentiation repertoire. The significance of the findings is three-fold, 1<sup>st</sup>) They validate the hypothesis that the BM stromal cells express a pluridifferentiated progenitor cell phenotype, providing insight into the molecular basis of cellular plasticity as well as establishing the utility of single-cell genomics, 2<sup>nd</sup>) They provide evidence for a common progenitor for mesenchymal progenitors and BM-derived (B) lymphoid progenitors, 3<sup>rd</sup>) By establishing a comprehensive phenotype of cultured bone marrow stromal cells at single cell level for the first time, the findings pave the road for ultimate identification and investigation of these cells in fresh samples of marrow, normal as well as diseased, in which they occur at a low frequency.

## MATERIALS & METHODS

### Second-Tier Data-Analysis/Data Mining

The microarray data outputted by MAS v.5 (in the form of tab delimited text files) were imported into GENESPRING software version 4.2.1 (Silicon Genetics, Redwood City, Calif.). Following instructions accompanying GENESPRING, each gene was normalized to itself (per-gene normalization) by using the median of the gene's expression values over all the samples of an experimental group (or groups) and dividing each measurement for that gene by the corresponding median value, assuming that it was at least 0.01. The ratios were then log transformed to base e. No per-sample normalization was performed in GENESPRING since it was already accomplished as part of MAS v.5 analysis. The purpose of the above data transformations, including scaling and normalization, was to remove systematic error within and across conditions or experimental groups prior to further analysis. GENESPRING was used to achieve the following data-analysis objectives.

a) Filtering for reliably present genes by eliminating the genes with weak expressions that are statistically close to the background estimate. As per the instructions accompanying GENESPRING, random error was estimated from control strength or median measurement level using the two-component global error model of Rocke-Lorenzato that assumes variability between replicates, as being similar for all genes showing similar measurement level. The formula for the error model of normalized expression levels can be written as follows:

$$S(\text{norm})^2 = a^2/C^2 + b^2$$

Where, S=standard error of normalized expression data, a & b are the two error components, a=an absolute or fixed error component impacting at lower measurement values, b=a relative or proportional error component impacting at higher

measurement levels, and C=control strength. According to the manufacturer, a curve is fitted for each group of replicates, with standard error of normalized data on Y-axis vs. control strength on X-axis. At lower end of control strength, the normalized standard error would be high and as the control strength increases, the standard error would decrease reaching a point where the curve flattens and data become more reliable. Control strength for each condition or sample group at which the above-referred two error components contribute equally, was calculated as follows, for collective MPCs, C=128.68; collective USCs, C=253.52; single-cell MPCs, C=348.32. Each condition was filtered for genes expressing signals greater than the respective control strength, thus eliminating the genes with weak expressions from each group. Note 7,196 genes (out of 12,625 gene probes tested) passed the restriction in case of cMPCs, 7,287 genes in case of cUSCs and 5,937 in case of sMPCs. Corresponding gene lists were created.

b) Filtering for genes “present” across replicates in each sample group. GENESPRING’s “Add data file restriction” feature was used to prepare the respective lists of genes that were present (or expressed or active) in a least 7 of 8 cUSC samples, 4 of 5 cMPC samples, and 9 of 10 sMPC samples. Note 5,204 genes (out of 12,625 gene probes tested) passed the restriction in case of cMPCs, 4,763 genes in case of cUSCs, and 3,124 genes in case of sMPCs. Corresponding gene lists were created.

c) Exclusion of genes with weak expressions from genes “present” in each sample group. Respective gene-lists for each sample group from steps (a) and (b), were intersected via Venn diagrams. Note 5,204 genes passed the restriction in case of cMPCs, 4,761 genes in case of cUSCs, and 3,124 genes in case of sMPCs, which are almost identical to the numbers obtained as under step (b), except for a difference of 2 genes in case of cUSCs, thus providing no significant improvement in restricting the data beyond under step (b). This is a reflection of the high stringency of the criterion employed under step (b). The 2 genes in case of cUSCs that passed the restriction under step (b) but failed the combined restriction under step (c) did show weak expressions (raw signals ranging, 142-331). Corresponding gene lists were created.

d) Preparation of master stromal cell gene list. Respective gene lists for the three sample groups from step (c) were intersected via Venn diagrams, resulting in identification of a list of 2,755 genes that are uniformly present or expressed in at least 20 of 23 stromal cell samples investigated. The stromal cell gene list thus arrived at contained genes that are representative of diverse mesenchymal lineages. Parenthetically, intersecting of gene lists corresponding to the three sample groups from step (b) resulted in a stromal cell gene list consisting of 2,756 genes, thus differing by 1 gene from the “official” master stromal cell gene list.

e) Two-way hierarchical clustering of 2755 stromal cell genes based on expression profiles in 23 stromal cell samples. Only the data that were “cleaned up” of genes with weak expressions as outlined under step (a) were used for hierarchical clustering. This necessitated further processing of data in MICROSOFT ACCESS prior to analysis by GENESPRING. Note that the data for each individual sample as outputted by MAS v.5 contained probe IDs, quantitative and qualitative data, as well as other information such as annotations and are readily recognizable by GENESPRING. In contrast, the gene list, resulting from step (a), contained only probe IDs and could not contain the data associated with each individual sample and was not recognizable by GENESPRING for inputting as part of an Experiment. Therefore,

the microarray data for each group of individual samples (in EXCEL format) as well as the corresponding gene list for that group from step (a) (also in EXCEL format) were imported into an ACCESS database. The genes that did not pass the test under step (a) were deleted from the microarray data for each individual sample by querying and intersecting with the appropriate post-clean-up gene list. The resulting data files were saved first as EXCEL files, then re-saved as tab delimited text files and then imported into GENESPRING. Per-gene normalization and log transformation were applied as described above. “Gene Tree” and “Experiment Tree” were constructed by applying a method similar to that of Eisen et al as implemented in GENESPRING and by using the stromal cell gene list and the following parameters: standard correlation as similarity measure; a minimum distance of 0.001; and a separation ratio of 0.5 in case of Gene Tree and 1.0 in case of Experiment Tree.

f) Preparation of stromal cell gene lists as relevant to different cellular phenotypes and/or functions. The gene lists associated with distinct mesenchymal cell lineages or phenotypes, i.e., osteoblasts, fibroblasts, muscle cells and adipocytes, etc., were prepared using a combination of methods. These include 1) Visually inspecting the entire stromal-cell gene list for relevant key words. 2) Directly searching the stromal cell gene list by using key words of interest via “Advanced Find Genes” feature under Edit menu in GENESPRING and by selecting “Search Only Current Gene List”. 3) Intersecting the stromal cell gene list with gene lists of interest from Gene Ontology lists, e.g., list of oncogenes, via Venn diagrams.

g) Visualization of gene-expression plots. The expression pattern of a gene across a given group (or groups) of samples of interest was pictured via Gene Inspector window, utilizing desired display options.

Throughout this application, various publications, are referenced by author and year. Full citations for the publications are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

The preceding descriptions of the invention are merely illustrative and should not be considered as limiting the scope of the invention in any way. From the foregoing description, one of ordinary skill in the art can easily ascertain the essential characteristics of the instant invention, and without departing from the spirit and scope thereof, can make various changes and/or modifications of the inventions to adapt it to various usages and conditions. As such, these changes and/or modifications are properly, equitably, and intended to be, within the full range of equivalence of the following claims.

#### Example 9

The nonhematopoietic stromal cells of the bone marrow are critical for the development of hematopoietic stem cells into functionally competent blood cells. This study addresses the question of whether bone marrow stromal cell cultures in the Dexter system propagate multiple different mesenchymal stromal cell types or one stromal cell type that expresses multiple phenotypes simultaneously. Results show that isolated single stromal cells simultaneously express transcripts associated with osteoblast, fibroblast, muscle, and adipocyte differentiation. Furthermore, isolated single stromal cells simultaneously express transcripts characteristic of epithelial



cells, endothelial cells and neural/glia cells. Isolated single stromal cells also express transcripts for CD45, CD19, CD10, CD79a, and representative proto-oncogenes and transcription factors, which are typically associated with normal and neoplastic hematopoietic cells. These findings suggest that the nonhematopoietic mesenchymal cells and the hematopoietic B-lymphocytes have a common progenitor. This is consistent with the idea that progenitor cells express genes that are characteristic of the multiple lineage paths that such cells may be capable of adopting. This study demonstrates the technical feasibility of transcriptome analysis of individual primary cell-culture grown stromal cells, and supports the concept that bone marrow stromal cells are relatively homogeneous and show a phenotypic signature of potential multilineage differentiation capacity.

As noted above, a recent study from the inventor's laboratory suggested the existence of a single unique pluridifferentiated stromal mesenchymal progenitor cell (MPC) type (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). However, the mesenchymal lineage markers used previously are difficult to assess in the same cell. A later study by Tremain et al. applied micro-serial analysis of gene expression (micro-SAGE) to determine the "transcriptome" of a single colony-forming "unit-fibroblast" derived from a population of mesenchymal stem cells (MSCs) from Friedenstein system (N. Tremain et al. *Stem Cells* 19 (2001) 408-418). These MSCs (that are relatively less differentiated in comparison to MPCs in Dexter system) also contained transcripts common to bone, cartilage, muscle, epithelium and neural cells, which supports the concept that BM stromal cells express a pluridifferentiated mesenchymal phenotype. However, the study by Tremain et al. (N. Tremain et al. *Stem Cells* 19 (2001) 408-418) only analyzed a single colony of BM fibroblasts, CFU-F, consisting of approximately 10,000 cells. Because such a large a colony of cells is not necessarily clonal, it could potentially contain multiple discrete singly differentiated mesenchymal cell-types. Another study examined a clonally-derived marrow stromal cell line that expressed the genes representative of all three germ layers (D. Woodbury et al. *J. Neurosci. Res.* 69 (2002) 908-917), supporting the idea of a pluridifferentiated stromal progenitor cell (Seshi, S. et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246).

The stromal cell—B cell relationship. Evidence for a progenitor cell common to stromal cells and hematopoietic cells has been emerging in fragments from isolated reports. Singer et al. in 1984 (J. W. Singer et al. *Leuk. Res.* 8 (1984) 535-545) while investigating bone marrow cultures from human patients with clonal myeloproliferative disorders showed that the nonhematopoietic stromal cells were derived from the same clonal progenitors that were involved by the hematopoietic neoplasm, revealed by G6PD marker analysis. Huss et al. in 1995 (R. Huss, et al. *Proc. Natl. Acad. Sci. U.S.A.* 92 (1995) 748-752) by studying a canine BM stromal cell line showed that the adherent stromal cells had "turned" into nonadherent hematopoietic cells, especially when the latter were cultured in the presence of stem cell factor. Pessina et al. in 1997 (A. Pessina, et al. *Exp. Hematol.* 25 (1997) 536-541) showed that a particular murine stromal cell line, upon stimulation with bFGF, expressed a B-cell phenotype, including CD45R and surface immunoglobulin. The present report shows for the first time that isolated single stromal cells express transcripts that are typically associated with hematopoietic lineage, namely CD45 and CD19, as well as relevant proto-oncogenes and transcription factors. These results strongly support the existence of a progenitor cell common to bone marrow stromal cells and hematopoietic cells, particularly the bone marrow-derived (B) lymphocytes. Even though

many of the above genes are not unique to B cells, the inventor's conclusions are not based on expression of any one gene. Simultaneous expression of a panel of genes (CD10+, CD19+, CD79A+, HLA-Dr+) is indeed unique to pre-B cells. To the inventor's knowledge, only pre-B cells and BM stromal cells express this composite phenotype.

The experiments presented here use gene expression analysis of isolated, single, primary, normal human bone marrow stromal cells, which are known to have a broad capacity for multilineage differentiation. The isolated cells that are the targets of the present analysis are pictured in FIG. 15. Progenitor cells express genes that are characteristic of any of the lineage fates that such cells are capable of adopting. Although conversion of stromal cells into hematopoietic or B cells has not been achieved, this work complements the work by earlier investigators outlined above (J. W. Singer et al. *Leuk. Res.* 8 (1984) 535-545; R. Huss et al. *Proc. Natl. Acad. Sci. U.S.A.* 92 (1995) 748-752; A. Pessina et al. *Exp. Hematol.* 25 (1997) 536-541) and provides new evidence involving gene expression patterns for possible lineage relationship between stromal cells and hematopoietic cells. In addition, this study may provide researchers with the tools and information to facilitate a search for cell culture conditions that permit development of B cells from an isolated single stromal cell.

Pluripotentiality vs. pluridifferentiation. A number of investigators have recently shown that hematopoietic stem cells and nonhematopoietic stem cells alike have the capability to transdifferentiate by turning stem cells into variety of tissues revealing their extraordinary pluripotentiality (M. F. Pittenger et al. *Science* 284 (1999) 143-147; C. R. Bjornson et al. *Science* 283 (1999) 534-537; M. A. Eglitis et al. *Proc. Natl. Acad. Sci. U.S.A.* 94 (1997) 4080-4085; T. R. Brazelton et al. *Science* 290 (2000) 1775-1779; I. Wilmot et al. [published erratum appears in *Nature* 1997 Mar. 13;386(6621):200], *Nature* 385 (1997) 810-813; D. S. Krause et al. *Cell* 105 (2001) 369-377; Y. Jiang et al. *Nature* 418 (2002) 41-49). The technical foundations of the studies that led to the excitement about transdifferentiation or plasticity of stem or progenitor cells have been recently vigorously challenged (N. Terada et al. *Nature* 416 (2002) 542-545; Q. L. Ying et al. *Nature* 416 (2002) 545-548; W. A. Wells *J. Cell Biol.* 157 (2002) 15-18; A. E. Wurmser et al. *Nature* 416 (2002) 485-487; C. Holden et al. *Science* 296 (2002) 2126-2129; R. Y. Tsai et al. *Dev Cell* 2 (2002) 707-712; K. Dorshkind *Nat Immunol* 3 (2002) 311-313; S. L. McKinney-Freeman et al. *Proc. Natl. Acad. Sci. U.S.A.* 99 (2002) 1341-1346). Two technical artifacts that could potentially provide misleading results are, a) donor cells can adopt the phenotype of other cells by spontaneous cell fusion, making them masquerade as transdifferentiated cells (N. Terada et al. *Nature* 416 (2002) 542-545; Q. L. Ying et al. *Nature* 416 (2002) 545-548) and b) heterogeneity of stem cell types that pre-exist within different tissues also can provide misleading results (K. Dorshkind et al. *Nat Immunol* 3 (2002) 311-313; S. L. McKinney-Freeman et al. *Proc. Natl. Acad. Sci. U.S.A.* 99 (2002) 1341-1346; S. H. Orkin et al. *Nat Immunol* 3 (2002) 323-328). As noted above, the present investigation involves isolated single stromal cells, specifically 10 cells from 4 different individuals (FIG. 15). The cell culture system has been earlier characterized at the light microscopic level, ultrastructural level and by karyotypic analysis; these analyses revealed no evidence for spontaneous cell fusion or stem cell heterogeneity (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). Spontaneous cell fusion most likely involves monocyte/macrophages forming multinucleated giant cells (N. Terada et al. *Nature* 416 (2002) 542-545); however, it was observed no expression of



myelomonocytic marker genes by isolated single stromal cells (Table 17). The interpretation of in vivo transdifferentiation studies involves localization of different lineage cells in different tissues or organs; such a situation only requires fusion between two cell types (one donor cell and one recipient cell) for investigators to believe the artifact as transdifferentiation. The probability of an array of different cell types fusing into one cell, which then masquerade as a pluridifferentiated cell, in 10 out of 10 cells studied, is very low.

Although numerous reviews exist on the technology of single-cell genomics, few studies have applied this technology (D. M. O'Dell et al. *Arch. Neurol.* 56 (1999) 1453-1456; P. B. Crino et al. *Proc. Natl. Acad. Sci. U.S.A.* 93 (1996) 14152-14157; J. Cossman *J. Histochem. Cytochem.* 49 (2001) 799-800; J. Eberwine *Nat. Neurosci.* 4 Suppl (2001) 1155-1156; N. N. Iscove et al. *Nat. Biotechnol.* 20 (2002) 940-943), and to the inventor's knowledge, this is the first report of successful application of the Affymetrix microarray analysis at the single cell level. These experiments were facilitated by the fact that sMPCs are uniquely large cells with abundant, tightly packed cytoplasm and conceivably contain relatively large amount of starting mRNA, as for example compared to a lymphocyte.

Investigators have shown that BM stromal cells under select culture conditions can be turned into bona fide bone cells, muscle cells, fat cells (M. F. Pittenger et al. *Science* 284 (1999) 143-147; S. Wakitani et al. *Muscle Nerve* 18 (1995) 1417-1426; S. E. Haynesworth et al. *Bone* 13 (1992) 81-88), glial cells (G. C. Kopen et al. *Proc. Natl. Acad. Sci. U.S.A.* 96 (1999) 10711-10716), and nerve cells (J. Sanchez-Ramos et al. *Exp. Neurol.* 164 (2000) 247-256; I. B. Black et al. *Blood Cells Mol. Dis.* 27 (2001) 632-636), demonstrating their pluripotentiality. By suggesting a molecular mechanism for stromal cell plasticity, the present data support the existence of a common precursor for MPC/neural and other lineages. These results provide an independent validation of the studies on transdifferentiation, such as the extraordinary multilineage potency of BM-derived stem or progenitor cells, reported by Krause's group (D. S. Krause et al. *Cell* 105 (2001) 369-377) and Verfaillie's group (Y. Jiang et al. *Nature* 418 (2002) 41-49). "Lineage burst" characterized by simultaneous activation of diverse differentiation pathways within the same cell appears to be the signature profile of the stromal cell, which indicates that a "pluripotent" cell is "pluridifferentiated" at least at the molecular level. These results also imply that conversion of a stromal progenitor cell into a terminally differentiated cell (such as bone cell, muscle cell, fat cell, fibroblast, etc.) would need to "turn off" the diverse cellular pathways that are simultaneously active in a stem or progenitor cell. A recent study showing a clonally-derived BM stromal cell line expressed the genes representative of all three germ layers (D. Woodbury et al. *J. Neurosci. Res.* 69 (2002) 908-917) provides independent support to the concept of a pluridifferentiated stromal progenitor cell (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). Support also comes from the observation that multilineage gene expression precedes unilineage commitment in the hematopoietic system (M. Hu et al. *Genes Dev.* 11 (1997) 774-785).

It is likely that the multipotential cells in the marrow are rare, occurring at an estimated frequency of 1 in  $10^4$  nucleated cells (M. Galotto et al. *Exp. Hematol.* 27 (1999) 1460-1466). However, these cells have been culture-expanded over 4 weeks. Cultured stromal cells represent the progeny of the stromal cell, and not necessarily the stromal cell itself, for which no in vivo assay as yet exists. The suggestion that nonhematopoietic mesenchymal cells and B-lymphocytes share a common precursor is based on expression of a panel of

genes (CD45+/-, CD34+/-, CD20+/-), (CD10+, CD19+, CD79A+, HLA-Dr+), and not expression of CD 19 alone. Similar ideas were expressed in a recent paper (K. Akashi et al. *Blood* 101 (2003) 383-389) and the accompanying commentary (T. Enver *Blood* 101 (2003) 381). While this paper reports that the hematopoietic stem cells of varying potential express the genes associated with a variety of nonhematopoietic cell types, the present study reports nonhematopoietic stromal progenitor cells which express the genes associated with hematopoietic cells, in particular B cells. These two reports raise the question as to how hematopoietic stem cells and nonhematopoietic stromal cells are related in terms of ontogeny.

#### Materials and Methods (Example 9)

The present study involved microarray analysis of 23 samples and a corresponding number of chips. The marrow samples were obtained from 4 normal healthy adult human subjects, and consisted of mixtures of unfractionated stromal cells (collective USCs or cUSCs, 8 samples), Percoll gradient-purified mesenchymal progenitor cells (collective MPCs or cMPCs, 5 samples) and single-cell MPCs (sMPCs, 10 samples) obtained by laser-capture microdissection (LCM) (M. R. Emmert-Buck et al. *Science* 274 (1996) 998-1001), ensuring adequate controls and replicates. The isolated single stromal cells were selected on the basis of morphology. Wright-Giemsa (or hematoxylin) stained cytospin preparation revealed characteristically large nonhematopoietic cells with a relatively irregular nucleus and cytoplasm compartmentalized into ectoplasm and endoplasm (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). Hematoxylin stain is simpler to use, provides morphologic detail sufficient to allow recognition and isolation of these cells by laser capture microdissection and does not interfere with downstream microarray testing. The photomicrographs of 10 stromal cells that have been subjected to microarray testing are shown in FIG. 15. As characterized earlier using immunocytochemical staining (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246), the stromal cells targeted for microarray analysis were CD45-negative cells, thus separating them from contaminating hematopoietic cells. To serve as controls and facilitate comparison, 8 samples of unfractionated stromal cells that were "contaminated" by up to 35% macrophages and 5% hematopoietic cells (cUSC), and 5 samples of Percoll-gradient purified stromal cells, up to 95% pure (cMPC) were analyzed side-by-side (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). RNA isolated from sMPC samples was subjected to 2 rounds of amplification using RiboAmp kit (Arcturus, Inc) before in vitro transcription (IVT). In contrast, RNA samples isolated from cUSCs and cMPCs were used without amplification for IVT. Except for this difference, the steps of microarray testing were standard for all 3 types of samples and are outlined as follows: Preparation of total RNA→generation of cDNA→preparation of ds cDNA→in vitro transcription into cRNA→fragmentation of cRNA→hybridization of target RNA to a microarray of known genes (Affymetrix U95Av2 oligonucleotide microarray, with 12,625 probe sets)→Signal quantification and first-tier analysis using the microarray quantification software (Microarray Suite MAS v.5, Affymetrix, Inc). According to the statistical expression analysis algorithm implemented in MAS v.5, the presence of a gene within a given sample was determined at a detection p-value of <0.05, and was graded absent (A), marginal (M) or present/positive (P).

#### Dexter-Type Bone Marrow Stromal Cell Culture

This study involved bone marrow samples obtained from four healthy adults (3 women and 1 man) ranging in age from

43-50 years. The subjects were qualified to donate bone marrow for transplantation in a standard clinical BMT setting. Stromal cells were cultured using BM mononuclear cells as the starting cells and following standard protocols as have been ongoing in this laboratory, i.e., in presence of hydrocortisone and horse serum (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246; B. Seshi *Blood* 83 (1994) 2399-2409; S. Gartner et al. *Proc. Natl. Acad. Sci. U.S.A.* 77 (1980) 4756-4759). The stromal cells representing mesenchymal progenitor cells (MPCs) (~95% pure), were purified or enriched as described using a discontinuous Percoll gradient after selective killing of the macrophages in stromal cultures with L-leucine methyl ester (LME, Sigma) (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). Detailed protocols used were published earlier (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). Briefly, the BM mononuclear cells were cultured for 4 weeks, monolayers (FIG. 1) were trypsinized, and nonhematopoietic cells were purified by Percoll gradient (FIG. 2) before they were cytopspun in preparation for laser-capture microdissection (LCM). All samples were treated identically. The unfractionated samples contained on average 40% contaminating cells (35% macrophages+5% hematopoietic cells) whereas Percoll gradient-enriched samples contained on average 5% contaminating cells (macrophages+hematopoietic cells).

#### Isolation of Individual MPCs Using Laser Capture Microdissection (LCM)

Strict laboratory precautions were observed to ensure preservation of RNA. All buffers and solutions, e.g., phosphate-buffered saline (PBS) and ethanol solutions contained DEPC-treated water. Before microdissection of individual stromal cells, cytopspins of Percoll-purified MPCs were prepared by attaching dispersed BM stromal cells to uncoated glass slides by low speed (400 rpm) cytocentrifugation using Shandon cytopspin centrifuge. The cytopspins were fixed in 95% ethanol for 10 min and stained for 30 sec using Hematoxylin QS (Vector, Burlingame, Calif.) followed by washing in DEPC water. The cytopspins were then dehydrated in increasing concentration of ethanol and treated in xylene. This is a single-step staining method without involving a bluing protocol; it provided sufficient morphologic detail and did not interfere with downstream microarray analysis. The MPCs selected on the basis of morphology, as visualized on the microscope monitor, were microdissected (M. R. Emmert-Buck et al. *Science* 274 (1996) 998-1001) using PixCell II (Arcturus, Inc) and captured on CapSure LCM Caps (Arcturus), followed by extraction of RNA (see next).

#### Microarray Sample Preparation and Testing

Unless mentioned otherwise, sample preparation and microarray testing were performed according to the protocols outlined in the GENECHIP Expression Analysis Technical Manual (Affymetrix, Inc, Santa Clara, Calif.). RNeasy mini protocol kit (Qiagen, Valencia, Calif.) was used for isolation of total RNA from unfractionated stromal cells (USCs) and from Percoll-purified MPCs. Superscript II cDNA synthesis kit (Invitrogen) primed with a T7-(dT)<sub>24</sub> primer containing a T7 RNA polymerase promoter sequence (Genset Oligos, La Jolla, Calif.) was employed to prepare ds cDNA from unamplified RNA samples, using 8-10 µg aliquots of total RNA as the template for first strand cDNA synthesis. The PicoPure RNA Isolation Kit (Arcturus, Mountain View, Calif.) was employed for extraction of RNA from LCM-dissected single-cell MPC samples. RiboAmp RNA amplification kit (Arcturus, Inc) (L. Luo, et al. *Nat. Med.* 5 (1999) 117-122) was used to amplify RNA from the LCM-dissected single cell samples by performing two rounds of amplification, terminating the

reaction after completion of ds cDNA synthesis. The entire amplified RNA sample was used as the template for cDNA synthesis. The following steps were identical for both unamplified and amplified RNA samples. In vitro transcription (IVT) was performed in the presence of biotinylated UTP and CTP to produce biotin-labeled cRNA (Bioarray High Yield RNA Transcript labeling Kit, Enzo Diagnostics, Inc, Farmingdale N.Y.), followed by cleaning of the reaction products with RNeasy Mini Kit columns (Qiagen, Valencia, Calif.). The purified, biotin-labeled cRNA samples were then submitted to the Microarray Core Facility at the University of Florida, Gainesville, where the following steps were performed. a) Controlled fragmentation of target cRNA in the presence of heat and Mg<sup>2+</sup> b) Hybridization of fragmented cRNA (15 µg) for 16 h at 45° C. to a microarray of known gene probes (U95Av2 oligonucleotide microarray, containing ~12,500 gene probes). c) Washing and staining of the probe array with SAPE (streptavidin phycoerythrin) (Molecular Probes, Inc, Eugene, Oreg.). d) Array scanning with an Agilent argon-ion laser equipped with 488 nm emission and 570 nm detection wavelengths (GENEARRAY Scanner). e) Background subtraction/signal quantification and first-tier analysis using the newest-version of the microarray quantification software, MICROARRAY Suite (MAS v.5, Affymetrix, Inc). That a gene within a given a sample was absent (A), marginal (M) or present/positive (P) was determined at a detection p-value of <0.05, according to the statistical expression analysis algorithm implemented in MAS v.5. First-tier analysis also included per chip (or per sample) normalization in MAS v.5 by scaling the trimmed mean signal of a probe-array to a constant target signal value of 2,500 to facilitate comparison of the results from different samples.

FIGS. 21A-21F shows gene-expression plots of representative precursor B-lymphocyte-associated genes by collective MPCs and single-cell MPCs. Individual samples are represented on X-axis. Signal intensity of a transcript in log scale (normalized across 15 samples) is shown on Y-axis. The CD markers that are traditionally associated with hematopoietic cells, CD45 (probe ID 40518\_at), CD19 (ID 1116\_at) and CD34 (ID 538\_at), are expressed by sMPCs. CD45, when present, is more abundantly detected in single MPCs than in collective MPCs, and is particularly noticeable by wide range of log scale for CD45. The other pre-B cell associated markers that are expressed by sMPCs are CD10 (ID 1389\_at), HLA-Dr (ID 33261\_at) and CD79A (ID 34391\_at). Samples 1-5, respectively, represent MPC A, MPC B R2, MPC C R2, MPC D R1, MPC D R2. Samples 6-15, respectively, represent SCA1, SCA2, SCA3, SCB1, SCB3, SCC1, SCC3, SCD1, SCD2, and SCD3.

FIG. 22A-22F shows scatter plots using log transformed data and showing systematic analysis of transcriptome wide random variation. The methods involved in construction of scatter plots are described in the section entitled, "Second-tier data-analysis/data mining". The results are discussed in the section entitled "Data mining and reproducibility of overall procedures".

#### Second-Tier Data-Analysis/Data Mining

The microarray data outputted by MAS v.5 (in the form of tab delimited text files) were imported into GENESPRING software version 4.2.1 (Silicon Genetics, Redwood City, Calif.).

Following instructions accompanying GENESPRING, each gene was normalized to itself (per-gene normalization) by using the median of the gene's expression values over all the samples of an experimental group (or groups) and dividing each measurement for that gene by the corresponding

median value, assuming that it was at least 0.01. The ratios were then log transformed to base e. No per-sample normalization was performed in GENESPRING because it was already done as part of MAS v.5 analysis. The purpose of the above data transformations, including scaling and normalization, was to remove systematic error within and across conditions or experimental groups. GENESPRING was used to achieve the following data-analysis objectives. a) Filtering for reliably present genes by eliminating the genes showing weak expressions statistically close to the background estimate. As per the instructions accompanying GENESPRING, random error was estimated from control strength or median measurement level using the two-component global error model of Rocke-Lorenzato that assumes variability between replicates as being similar for all genes showing similar measurement levels [49]. The formula for the error model of normalized expression levels may be written as follows:

$$S(\text{norm})^2 = a^2/C^2 + b^2$$

Where, S=standard error of normalized expression data, a & b are the two error components, a=an absolute or fixed error component impacting at lower measurement values, b=a relative or proportional error component impacting at higher measurement levels, and C=control strength. According to the manufacturer, a curve is fitted for each group of replicates, with standard error of normalized data on Y-axis vs. control strength on X-axis. At lower end of control strength, the normalized standard error would be high and as the control strength increases, the standard error would decrease reaching a point where the curve flattens and data become more reliable. Control strength for each condition or sample group, where  $C=a/b$ , at which the two error components contribute equally, was calculated as follows, for collective MPCs,  $C=128.68$ ; collective USCs,  $C=253.52$ ; single-cell MPCs,  $C=348.32$ . Each condition was filtered for genes expressing signals greater than the respective control strength, thus eliminating the genes with weak expressions from each group. Of 12,625 gene probes tested, 7,196 genes passed the restriction in case of cMPCs, 7,287 genes in case of cUSCs and 5,937 in case of sMPCs. Corresponding gene lists were created. b) Filtering for genes “present” across replicates in each sample group. GENESPRING’s “Add data file restriction” feature was used to prepare the respective lists of genes that were present (or expressed or active) in at least 7 of 8 cUSC samples, 4 of 5 cMPC samples, and 9 of 10 sMPC samples. Of 12,625 genes tested, 5,204 genes passed the restriction in case of cMPCs, 4,763 genes in case of cUSCs, and 3,124 genes in case of sMPCs. Corresponding gene lists were created. c) Exclusion of genes with weak expressions from genes “present” in each sample group. Respective gene-lists for each sample group from steps (a) and (b), were intersected via Venn diagrams. As a result, 5,204 genes passed the restriction in case of cMPCs, 4,761 genes in case of cUSCs, and 3,124 genes in case of sMPCs, which are almost identical to the numbers obtained as under step (b), except for a difference of 2 genes in case of cUSCs, thus providing no significant improvement in restricting the data beyond under step (b). This is a reflection of the high stringency of the criterion used under step (b). The 2 genes in case of cUSCs that passed the restriction under step (b) but failed the combined restriction under step (c) did show weak expressions (raw signals ranging, 142-331). Corresponding gene lists were created. d) Preparation of the master list of stromal cell genes. Respective gene lists for the three sample groups from step (c) were intersected via Venn diagrams, resulting in identification of a list of 2,755 genes that are uniformly

present or expressed in at least 20 of 23 stromal cell samples investigated. The stromal cell gene list thus arrived at contained genes that are representative of diverse mesenchymal lineages. Parenthetically, intersecting of gene lists corresponding to the three sample groups from step (b) resulted in a stromal cell gene list consisting of 2,756 genes, thus differing by 1 gene from the master list of stromal cell genes. e) Two-way hierarchical clustering of 2,755 stromal cell genes based on expression profiles in 23 stromal cell samples. Only the data that were “cleaned up” of genes with weak expressions as outlined under step (a) were used for hierarchical clustering. This necessitated further processing of data in MICROSOFT ACCESS before analysis using GENESPRING.

f) The data for each individual sample as outputted by MAS v.5 contained probe IDs, quantitative and qualitative data, as well as other information such as annotations and are readily recognizable by GENESPRING. In contrast, the gene list, resulting from step (a), contained only probe IDs and could not contain the data associated with each individual sample and was not recognizable by GENESPRING for inputting as part of an Experiment. Therefore, the microarray data for each group of individual samples (in EXCEL format) as well as the corresponding gene list for that group from step (a) (also in EXCEL format) were imported into an ACCESS database. The genes that did not pass the test under step (a) were deleted from the microarray data for each individual sample by querying and intersecting with the appropriate post-clean-up gene list. The resulting data files were saved first as EXCEL files, then re-saved as tab delimited text files and then imported into GENESPRING as modified experiments. Per-gene normalization and log transformation were applied as described above. “Gene Tree” and “Experiment Tree” were constructed by applying a method similar to that of Eisen et al. (M. B. Eisen et al. *Proc. Natl. Acad. Sci. U.S.A.* 95 (1998) 14863-14868) as implemented in GENESPRING and by using the stromal cell gene list and the following parameters: standard correlation as similarity measure; a minimum distance of 0.001; and a separation ratio of 0.5 in case of Gene Tree and 1.0 in case of Experiment Tree. f) Preparation of stromal cell gene lists as relevant to different cellular phenotypes and/or functions. The gene lists associated with distinct mesenchymal cell lineages or phenotypes, i.e., osteoblasts, fibroblasts, muscle cells and adipocytes, etc., were prepared using a combination of methods. These include 1) Visually inspecting the entire stromal-cell gene list for relevant key words. 2) Directly searching the master list of stromal cell genes by using key words of interest via “Advanced Find Genes” feature under Edit menu in GENESPRING and by selecting “Search Only Current Gene List”. 3) Intersecting the stromal cell gene list with gene lists of interest from Gene Ontology lists, e.g., list of oncogenes, via Venn diagrams. g) Visualization of gene-expression plots. The expression pattern of a gene across a given group (or groups) of samples of interest was pictured via Gene Inspector window, utilizing desired display options. h) Statistical analysis of random variation in expression of the master list of stromal cell genes vs. the complete list of genes tested. The master list of stromal cell genes with probe IDs from step (d) was imported into MICROSOFT ACCESS and intersected with the table containing complete Affymetrix primary data sets (Table 24). The resulting file was exported as EXCEL file consisting of the master list of stromal cell genes with the associated Affy data (Table 23). The Affy data as outputted by MAS v.5 (in the form of EXCEL Tables 23 and 24) were then imported into ARRAYSTAT software, Version 1.0, Rev.2.0 (Imaging Research Inc, St. Catharines, ON, Canada). The

data for each group of samples were log transformed to base 10, which allowed the software to construct the scatter plots, standard deviation vs. mean (FIGS. 22A-22F). i) Calculation of basic statistics for different sample groups. The mean and SD values presented as part of Tables 22A-D, 23 and 24 were calculated using MCG ARRAYSTAT Program (Richard A. McIndoe, URL: <http://www.genomics.mcg.edu/niddkbtc/Software.htm>). The accuracy of the reported mean and SD values was checked using EXCEL program.

#### Morphologic and Phenotypic Characterization of Cell Populations in Dexter Cultures

BM stromal cell cultures grown under Dexter conditions (i.e., in the presence of hydrocortisone and horse serum) are generally considered to be heterogeneous. Earlier published work, however, showed that Dexter cultures are not heterogeneous based on light microscopic, ultrastructural, phenotypic and molecular biological characteristics of the nonhematopoietic stromal cells isolated from these cultures (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246). Detailed characteristics of constituent cell populations in Dexter cultures were published previously (B. Seshi et al. *Blood Cells Mol. Dis.* 26 (2000) 234-246) and show that nonhematopoietic stromal cells (sMPCs) are morphologically and phenotypically uniform. Their morphologic characteristics are summarized as follows: The sMPCs are large cells with a relatively large irregular nucleus and abundant cytoplasm that is uniquely compartmentalized into ectoplasm and endoplasm. Macrophages are large cells as well, however they have a very small round bullet-like nucleus and foamy cytoplasm. In contrast, hematopoietic cells are small cells with minimal amount of cytoplasm. Earlier study used Wright-Giemsa stain. Comparable data are presented here using hematoxylin staining (FIG. 15) before laser-capture microdissection (LCM). To further characterize these cells, a Percoll-gradient technique was devised for enrichment of nonhematopoietic stromal cells (FIGS. 1 and 2, under Detailed Materials & Methods). While the unfractionated samples contained on average 40% contaminating cells (35% macrophages+5% hematopoietic cells), the Percoll gradient-enriched samples contained on average 5% contamination (macrophages+hematopoietic cells).

#### Assessment of RNA Amplification Method

The single-cell microarray data were reviewed for reproducibility and validity. Two important statistics, reflecting on the reliability of the RNA amplification step, were evaluated. a) 3': 5' ratios of housekeeping control genes: As shown in Table 5, these ratios were close to 1 in the standard unamplified samples, but were increased in the amplified samples. This may reflect preferential amplification toward 3' end since amplification may not proceed all the way to the 5' end. Alternatively, it may reflect sample degradation. The 3': 5' ratios were highly variable across single cell MPC samples in the case of ACTB (beta actin), but were relatively close in the case of GAPD (glyceraldehyde 3-phosphate dehydrogenase), suggesting that sample degradation did not occur. Furthermore, both GAPD and ACTB gene probes used as part of the standard gene probe set yielded relatively stable signals across replicates in each sample type, providing further evidence of intactness of RNA samples targeted for microarray analysis. b) Number of genes present or detected: As outlined in Table 5, the amplified single cell MPC RNA samples expectedly showed significantly lower numbers of genes compared to the standard RNA samples (average 34% vs. 46% of the genes etched on the array). The fact that the number of genes present is relatively constant across single cell replicate samples is further indication of the data reliability.

ity. DNA contamination was unlikely because of the RNA amplification method (which involved Oligo dT-based priming, T7 RNA polymerase-based RNA amplification, and DNase treatment of RNA samples before their purification). Of the 67AFFX hybridization and housekeeping positive control gene probe sets present on the U95A v2 chip, 22 genes were detected in at least 7 of 8 cUSC samples, 24 genes were detected in 4 of 5 cMPC samples and 19 genes were detected in at least 9 of 10 sMPC samples. Thirteen of these genes were present in at least 20 of the 23 samples investigated (Table 20). Similarly, stromal derived factors, SDF1, SDF2 and SDFR1 were detected in at least 20 of the 23 samples studied (Table 21).

#### Data Mining and Reproducibility of Overall Procedures

In many cases, microarray analysis is used to identify genes differentially expressed in different sample groups, (i.e., treated vs. untreated, or normal vs. diseased). In contrast, the goal in this study is to identify genes that are relatively uniformly expressed across normal untreated bone marrow stromal cell samples, regardless whether they are of single cell type or populations of cells, purified or unpurified. As described under Materials & Methods, GENESPRING has been used to achieve the following data-analysis objectives: a) Filtering for genes reliably detected in each sample group by eliminating the genes showing weak expression statistically close to the background estimate. b) Filtering for genes that are positive (present) across replicates in each sample group. c) Exclusion of genes with weak expression from genes present in each sample group. d) Preparation of a master list of stromal cell genes by intersecting gene lists from step (c) (FIG. 16). These steps have led to identification of a list of 2,755 genes that are detected in at least 7 of 8 cUSC samples AND 4 of 5 cMPC samples AND 9 of 10 sMPC samples (i.e., in at least 20 of 23 stromal cell samples investigated). The main conclusions of the present report are based on what is referred to as "master list of stromal cell genes" that is broadly representative of all 3 types of stromal cell samples investigated, and not on a gene list that is selective to sMPCs.

Not all of the 2,755 positive probes are non-redundant, as there are multiple probes for many individual genes on the chip employed. Since it was not possible to determine the actual number of genes that they represent, probe sets and genes are used interchangeably. The list of 2,755 genes in the "master list of stromal cell genes" represents 88% of the genes expressed by single cells (3,124); 58% of genes expressed by unfractionated samples (4,761); and 53% of genes expressed by Percoll-enriched samples (5,204). The remaining genes expressed by collective cell samples are probably due to contaminating cells as well as to genes whose transcripts failed to be amplified in single cell samples by the amplification method. As indicated in the preceding section, the amplified single-cell samples detected only ~34% of the genes tested (12,625), as opposed to unamplified cell samples which detected about 46% of the genes tested. The "remaining genes" list contains genes associated with myelomonocytic cells, which is consistent with contaminating cells. The "remaining genes" list also includes a number of mesenchymal-associated and other genes that failed to be amplified. As previously indicated, the enriched samples contained only 5% contaminating cells as opposed to unfractionated stromal cell samples which contained 40% contamination. In light of the high sensitivity of microarray analysis, 5% contamination is probably still sufficient to detect some genes associated with the contaminating cells. Contamination is recognized as a confounding factor in the analysis of gene expression results

involving populations of cells, however single cell expression profiling, as used here, is free from this artifact.

Hierarchical clustering analysis was used to construct a bone marrow stromal cell tree for visualizing global gene expression patterns across replicates and conditions. As shown in FIG. 17, stromal cell genes that are expressed at a relatively low level in amplified samples (sMPCs) are clustered to the left of the gene tree; genes that are more strongly expressed in sMPCs are prominently configured in the middle of the gene tree; and genes that are expressed approximately at the same level as unamplified samples (cMPCs and cUSCs) are clustered to the right of the gene tree. Most importantly, as evident on the sample or experiment tree, hierarchical clustering segregated the members of each sample type into a separate group (cMPC, cUSC and sMPC). Within each sample type corresponding subject replicates clustered together (with minor exceptions). This suggests a fairly high level of reproducibility within the data set.

The data in FIG. 18 show that different transcripts amplify to different extents. The expression of genes within the stromal cell gene list ranges from 0.2 to 6 (on the log scale) in unamplified samples (cMPC and cUSC) and from 0.02 to 36 in amplified samples (sMPC), thus showing much greater variability in the amplified samples. The effect of differential amplification is represented graphically using color-coding. Gene expression curves are colored (following the linear color bar shown on the right) according to the gene expression level in a particular single-cell sample, SCA1. The genes detected at a low level in this sample (as indicated in blue) are not necessarily expressed at a low level in unamplified samples (as read by the log scale on Y-axis). In fact, a significant number of them are expressed at a high level in the unamplified samples. This finding together with the observation that amplified samples detected about 34% of genes as opposed to unamplified samples detecting about 46% of genes tested (Table 5), suggests that some genes do not amplify at all by the method used, whereas other genes amplify to a sufficient degree to be detectable (shown in blue), while some other genes amplify to a degree equal to (in yellow) or surpassing (in red) the amounts in the collective samples. (The curve shown in white is the housekeeping gene, GAPD.) The statistical algorithm utilized in the latest version of Microarray Analysis Suite (MAS v.5) determined that a gene within a given sample was positive, regardless of grading.

Because different transcripts amplify variably, it is not possible to make a quantitative comparison across transcripts involving the amplified products. However, this does not preclude the usefulness of the amplification method for quantitative comparison of a particular transcript across amplified single cell samples. In fact, the data points of a given expression-curve in FIG. 18 are comparable within the amplified samples, suggesting that expression of a particular gene can be compared in different samples (i.e., normal vs. disease-associated MPCs). The reproducibility and the fidelity of linear amplification have been characterized previously (R. Raja, R. Salunga, T. Taylor, A. Bennett, A. Firouzi, A. Mennis, X.-J. Ma, D. Sgroi, M. Erlander, S. Kunitake, A microgenomics platform for high-throughput gene expression analysis of pure cell populations, *Journal of Clinical Ligand Assay* (in press) (2003)). It was observed that the spot intensities between replicate amplified samples showed a correlation of  $r=0.959$  and that amplified and unamplified gene expression ratios of mouse testis/brain showed a correlation of  $r=0.913$  (R. Raja, R. Salunga, T. Taylor, A. Bennett, A. Firouzi, A. Mennis, X.-J. Ma, D. Sgroi, M. Erlander, S. Kunitake, A microgenomics platform for high-throughput gene

expression analysis of pure cell populations, *Journal of Clinical Ligand Assay* (in press) (2003)). These findings suggest that quantitative comparison of differential gene expression is possible in cases where some but not all RNA samples are amplified.

Documentation of Statistical Variation in Expression of the Master List of Stromal Cell Genes vs. the Complete List of Genes Present on the Chip

There are multiple ways in which the genes of interest can be selected for further study after microarray testing. As outlined above, the stromal cell genes in the master list were selected on the basis of their positive calls in at least 20 out of 23 samples investigated. By plotting the mean expression levels vs. the standard deviation of the log transformed data, the statistical relationship between the expression levels vs. the background variation was determined for the master list of genes, and for the complete list of genes tested. As shown in FIGS. 22A-22F, the overall variation in the complete list of genes showed a negative trend with decrease in the variation as the mean signal strength increased. This result was observed with all three types of samples investigated. In contrast, similar plots involving the master list of stromal cell genes showed flat curves with the random variation or error being relatively constant, suggesting greater reliability of their measurements. Also, majority of genes with weak expression have been excluded from the master list, as evident from contrasting the mean expression levels shown on the horizontal axes for complete list of genes vs. master list of genes for all three types of samples. These observations would agree with the fact that the genes within the master list were to begin with uniformly present or expressed in at least 20 out of 23 samples tested.

Multilineage Gene Expression in Single Stromal Cells

A stromal cell gene list, generated as outlined above, is expected to be representative of typical stromal cell gene expression profile. Such master list of genes forms the basis for derivation of all other stromal cell gene lists, organized in accordance with lineage or functional categories. As depicted in FIGS. 19 & 20, and outlined in Table 18 (A-D), these findings show that isolated single cells simultaneously express genes associated with diverse mesenchymal cell lineages (namely, osteoblast, muscle, fibroblast and adipocyte), suggesting the existence of a pluridifferentiated mesenchymal progenitor cell type. An alternative interpretation of these findings is that the sensitive amplification/microarray approach detects levels of transcripts that are not physiologically relevant and may therefore detect 'leaky' transcriptional regulation in these cells. While "leaky" transcriptional regulation is possible, it is unlikely to be the case with sMPCs because the genes that formed the major basis for the foregoing conclusions are active not only in the amplified samples but also in the unamplified samples, ensuring that the results were not unduly biased by low level expression occurring only in the single cell samples.

As evident from the other gene lists (Table 18, E-G), an isolated single stromal cell simultaneously expresses transcripts for epithelial, endothelial and neural cell types as well, widening its transcriptomic repertoire. Furthermore, as shown in FIG. 21, and Table 18, H-J and Table 17, an isolated single stromal cell expresses transcripts that are typical of hematopoietic cells, in particular precursor B cells. This result supports the idea that the MPCs within the Dexter system might represent a form or stage of the progenitor cell that is common to nonhematopoietic and hematopoietic cells. That BM stromal cells express CD10 (CALLA) is not novel since BM stromal cells (A. Keating et al. *Br. J. Haematol.* 55

(1983) 623-628) as well as endometrial stromal cells (V. P. Sumathi et al. *J. Clin. Pathol.* 55 (2002) 391-392) and normal breast myoepithelial cells (S. Moritani et al. *Mod. Pathol.* 15 (2002) 397-405) are known to express CD10. However, the simultaneous expression of CD19, CD79A and immunoglobulin enhancer binding factors E12/E47 (proto-oncogene TCF3) by BM stromal cells is an unforeseen finding, and forms the basis for postulating the existence of a common progenitor with B cell lineage. B-cell progenitors typically display the phenotype, (CD45+/-, CD34+/-, CD20+/-), (CD10+, CD19+, CD79A+, HLA-Dr+), which is also displayed by isolated single stromal cells at least at the transcriptome level (Table 17). Primitive CD34+B cell precursors (so-called Whitlock-Witte initiating cells (C. A. Whitlock et al. *Proc. Natl. Acad. Sci. U.S.A.* 79 (1982) 3608-3612)) express the human homolog of the *Drosophila Polycomb* group gene, BMI1 that appears to be essential for the maintenance and proliferation of hematopoietic stem cells (J. Lessard et al. *Blood* 91 (1998) 1216-1224; J. Lessard et al. *Genes Dev.* 13 (1999) 2691-2703). As reported here, isolated single stromal cells also express BMI1 gene (Table 17 and Table 14).

CD45 positivity by cMPC and cUSC samples is attributable to coexisting or "contaminating" hematopoietic cells in these samples as evidenced by concurrent positivity for myelomonocytic markers CD13, CD33 and CD14 (Table 17). However, a similar explanation cannot be used in the case of isolated single stromal cells. Despite expression of numerous myeloid-associated proto-oncogenes and transcription factors, none of the typical myelomonocytic markers (e.g., CD13, CD33 and CD14) was identified in isolated single stromal cells. Similarly, other than CD4, no typical pan T cell lineage markers (e.g., CD5 and CD7) were detected in stromal cells. CD3 alpha and beta genes were not part of the gene chip used and therefore not tested.

The protein products or transcripts for CD45 and CD19 are most likely present in stromal cells at a basal level that is beyond the detection limits of conventional techniques, e.g., immunocytochemistry and Northern blotting. Correlation between transcriptome and proteome is estimated to be 0.48-0.76 (S. Hubbard, Functional genomics and bioinformatics, <http://www.bi.umist.ac.uk/users/mjfsjh/OPT-GNO/handout2001.htm> (2001)), accounting for the discrepancy in findings by conventional techniques vs. sensitive amplification/microarray analysis. Conceivably, two rounds of amplification prior to IVT sufficiently increased their transcript levels to be detected by microarray analysis. In fact, the CD45 levels were several-fold lower in cMPC and cUSC compared to CD45 levels in sMPC, and CD19 was undetectable in unamplified samples. Note that CD45 and CD19 are not isolated examples in this regard, since the inventor has identified at least 200 other genes that are uniquely present across sMPC samples but undetectable in cMPC and cUSC (see red circle, FIG. 16). These findings could alternatively be interpreted as evidence of lack of fidelity of the amplification method. However, 200 genes represent 1.58% the total genes tested (12,625) and 4.67% of the 4,283 genes (on average) detected in the amplified samples. Even assuming this alternative interpretation is correct, the fidelity of amplification measures over 95%. Only one gene was used, namely CD19, from the list of genes selective to sMPCs. Even if CD19 were excluded from consideration, the conclusions would still remain unchanged since they are not based on expression of any one particular gene but rather on simultaneous expression of a panel of lineage-associated genes.

Finally, the master list of stromal cell genes contained as many as 66 human homologs of *Drosophila*/homeotic genes.

Some of these genes are ubiquitously expressed in *Drosophila*, whereas other genes are known for their association with specific cellular pathways. As shown in Table 19, the human homologs of *Drosophila* genes, representing diverse cellular pathways, are simultaneously active in a stromal cell. This finding represents additional evidence supporting the existence of a pluridifferentiated mesenchymal progenitor cell type.

The gene lists disclosed herein are sub-lists of the master list of stromal cell genes consisting of 2,755 genes. Detailed lineage-associated gene lists (Tables 6-12 and Tables 14-16) as well as the master list of stromal cell genes with the associated Affymetrix primary data (Table 23). Expression of no one gene defines the phenotype of a particular cell type. Simultaneous expression of a panel of lineage related-genes in single isolated cell may be viewed as the harbinger of a potential cell type. Representative examples of genes corresponding to each cell lineage are outlined in this table

The stromal cell gene list contains a number of genes that are potentially capable of causing endothelial differentiation and vasculogenesis within the marrow microenvironment; however, these genes may not necessarily be endothelial cell markers. In fact, stromal cells express a gene, EDF1, the expression of which inversely correlates with endothelial cell differentiation within the stromal cells, indicating that the endothelial cell pathway is being actively "turned off" in these cells.

Despite expression of numerous myeloid-associated proto-oncogenes and transcription factors, none of the typical myelomonocytic markers (e.g., CD13, CD33 and CD14) was identified in stromal cells.

Despite expression of T-cell leukemia associated proto-oncogenes/transcription factors, no typical pan T-cell lineage markers (e.g., CD5 and CD7), other than CD4 and occasional CD2 and CD3 epsilon, were identified in stromal cells. CD3 alpha and beta genes were not part of the gene chip used and therefore not tested.

As noted previously, Table 17 lists stromal cells showing expression of genes typically associated with B-cell progenitors. Genes marked with asterisk (\*) met the criteria for inclusion in the master list of stromal-cell genes. Table 17 also shows that typical myelomonocytic markers (e.g., CD13, CD33 and CD14), and typical pan T-cell lineage markers (e.g., CD5 and CD7) were not detected in single stromal cells, except for occasional CD2 and CD3 epsilon. The Affymetrix primary data corresponding to the genes listed in Table 17 can be found in Tables 22A-D.

A list of human homologs of *Drosophila* genes, outlining more detailed descriptions of possible lineage associations is presented as an EXCEL file (Table 13). The Affymetrix primary data corresponding to the genes listed in Table 19 and Table 13 can be found in Table 23.

All patents, patent applications, provisional applications, and publications referred to or cited herein, whether supra or infra, are incorporated by reference in their entirety, including all figures, tables, nucleic acid sequence, amino acid sequences, and claims, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

## Tables

TABLE 5

Summary of human bone marrow stromal cell samples targeted for microarray analysis with an outline of the corresponding indicators of assay quality performance.			
	Unfractionated stromal cells (Collective USC, 8 replicates)	Percoll gradient-enriched stromal cells (Collective MPC, 5 replicates)	LCM-dissected single cell samples (Single Cell MPC, 10 replicates)
Subject A	UNFR A	MPC A	SCA1, SCA2, SCA3
Subject B	UNFR B, UNFR B R1, UNFR B R2	MPC B R2	SCB1, SCB3
Subject C	UNFR C R1, UNFR C R2	MPC C R2	SCC1, SCC3
Subject D	UNFR D R1, UNFR D R2	MPC D R1, MPC D R2	SCD1, SCD2, SCD3
Amplification of RNA before IVT	No	No	Two rounds
Number of genes present (% of 12,625)	Mean: 46.63 SD: 5.95	Mean: 46.54 SD: 3.66	Mean: 33.93 SD: 3.94
3':5' ratio, GAPD	Mean: 0.89 SD: 0.33	Mean: 1.23 SD: 0.53	Mean: 6.76 SD: 2.97
M33197 (Probe used as part of housekeeping control gene probe set)			
GAPD signal	Mean: 130,723 SD: 36,990	Mean: 164,587 SD: 40,204 (See FIG. 6B.)	Mean: 43,235 SD: 14,413 (See FIG. 6B.)
35905_s_at (Probe used as part of standard gene probe set)			
3':5' ratio, ACTB	Mean: 1.44 SD: 0.60	Mean: 2.29 SD: 1.57	Mean: 57.92 SD: 67.82
X00351 (Probe used as part of housekeeping control gene probe set)			
ACTB signal	Mean: 86,104 SD: 18,458	Mean: 100,383 SD: 28,427 (See FIG. 6B.)	◆Mean: 4,445 ◆SD: 884 (See FIG. 6B.)
32318_s_at (Probe used as part of standard gene probe set)			

## Footnote to Table 5

Replicate samples shown under each sample type as indicated correspond to each subject. The replicates of collective USC and collective MPC represent cell-culture or biological replicates of stromal cells grown in parallel flasks (instead of technical replicates). Of 27 samples, 2 collective MPC samples and 2 single cell MPC samples failed either at the test chip stage or produced unusual results in terms of the number of genes present and/or 3':5' ratios and were therefore excluded as outliers. The remaining 23 samples that were targeted for the data mining analysis are represented in this table. The statistics shown (means and SDs) were based on the number of sample replicates indicated in the top row of the table except for ACTB-signal for single cell MPCs (noted in the table by ◆), which were based on 9 replicates instead of 10.

TABLE 6

Osteoblast/bone cell/bone disorders (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
38112_g_at	CSPG2	X15998	chondroitin sulfate proteoglycan 2 (versican)	
38111_at	CSPG2	X15998	chondroitin sulfate proteoglycan 2 (versican)	
38126_at	BGN	J04599	biglycan	
36976_at	CDH11	D21255	cadherin 11, type 2, OB-cadherin (osteoblast)	
37315_f_at	BM036	AI057607	uncharacterized bone marrow protein BM036	
36996_at	OS-9	U41635	amplified in osteosarcoma	
41202_s_at	OS4	AF000152	conserved gene amplified in osteosarcoma	
671_at	SPARC	J03040	secreted protein, acidic, cysteine-rich (osteonectin)	
2087_s_at	CDH11	D21254	cadherin 11, type 2, OB-cadherin (osteoblast)	
1916_s_at	c-fos	V01512	Human cellular oncogene c-fos (complete sequence).	OMIM Notes: c-fos restricted to perichondrial growth regions of the cartilaginous skeleton.
1915_s_at	c-fos	V01512	Human cellular oncogene c-fos (complete sequence).	

TABLE 6-continued

<u>Osteoblast/bone cell/bone disorders (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
1388_g_at	VDR	J03258	vitamin D (1,25-dihydroxyvitamin D3) receptor	?Osteoporosis, involutional; Rickets, vitamin D-resistant
1451_s_at	OSF-2	D13666	osteoblast specific factor 2 (fasciclin I-like)	
32094_at	CHST3	AB017915	carbohydrate (chondroitin 6) sulfotransferase 3	
32307_s_at	COL1A2	V00503	collagen, type I, alpha 2	Ehlers-Danlos syndrome, type VIIA2; Marfan syndrome, atypical; Osteogenesis imperfecta, 3 clinical forms, 166200, 166210; Osteoporosis, idiopathic
32306_g_at	COL1A2	J03464	collagen, type I, alpha 2	Ehlers-Danlos syndrome, type VIIA2; Marfan syndrome, atypical; Osteogenesis imperfecta, 3 clinical forms, 166200, 166210; Osteoporosis, idiopathic
32305_at	COL1A2	J03464	collagen, type I, alpha 2	Ehlers-Danlos syndrome, type VIIA2; Marfan syndrome, atypical; Osteogenesis imperfecta, 3 clinical forms, 166200, 166210; Osteoporosis, idiopathic
34321_i_at	GS3786	D87120	predicted osteoblast protein	
34342_s_at	SPP1	AF052124	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	
34763_at	CSPG6	AF020043	chondroitin sulfate proteoglycan 6 (bamacan)	
222_at	EXT1	S79639	exostoses (multiple) 1	Chondrosarcoma; Exostoses, multiple, type 1
36822_at	WAC	U51334	WW domain-containing adapter with a coiled-coil region	Chondrosarcoma, extraskeletal myxoid
41202_s_at	OS4	AF000152	conserved gene amplified in osteosarcoma	
40790_at	BHLHB2	AB004066	basic helix-loop-helix domain containing, class B, 2; OMIM Notes: Alternative title, DEC1, expressed primarily in differentiated chondrocytes.	/transcription factor

TABLE 7

<u>Muscle/muscle disorders (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
38251_at	MLC1SA	AI127424	myosin light chain 1 slow a	
38923_at	FRG1	L76159	FSHD (Facioscapulohumoral muscular dystrophy) region gene 1	
37012_at	CAPZB	U03271	capping protein (actin filament) muscle Z-line, beta	
37279_at	GEM	U10550	GTP binding protein overexpressed in skeletal muscle	/GTPase
36791_g_at	TPM1	M19267	tropomyosin 1 (alpha)	Cardiomyopathy, familial hypertrophic, 3
36790_at	TPM1	M19267	tropomyosin 1 (alpha)	Cardiomyopathy, familial hypertrophic, 3
36792_at	TPM1	Z24727	tropomyosin 1 (alpha)	
36678_at	TAGLN2	D21261	transgelin 2	
36641_at	CAPZA2	U03851	capping protein (actin filament) muscle Z-line, alpha 2	
36931_at	TAGLN	M95787	transgelin	
37631_at	MYO1E	U14391	myosin 1E	
41439_at	MYO1B	AJ001381	myosin 1B	
40910_at	CAPZA1	U56637	capping protein (actin filament) muscle Z-line, alpha 1	/binds barbed ends of actin filaments



TABLE 7-continued

Muscle/muscle disorders (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
41187_at	MLC-B	U26162	myosin regulatory light chain	
41747_s_at	MEF2A	U49020	Human myocyte-specific enhancer factor 2A (MEF2A) gene, last coding exon, and complete cds.	
41738_at	CALD1	M64110	caldesmon 1	
41739_s_at	CALD1	M83216	caldesmon 1	
39791_at	ATP2A2	M23114	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Darier disease
39790_at	ATP2A2	M23115	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Darier disease
39378_at	BECN1	U17999	beclin 1 (coiled-coil, myosin-like BCL2 interacting protein)	
40488_at	DMD	M18533	dystrophin (muscular dystrophy, Duchenne and Becker types)	Becker muscular dystrophy; Cardiomyopathy, dilated, X-linked; Duchenne muscular dystrophy
40438_at	PPP1R12A	D87930	protein (myosin) phosphatase 1, regulatory (inhibitor) subunit 12A	OMIM Notes: Regulates the interaction of actin and myosin downstream of the guanosine triphosphatase Rho.
32838_at	smooth muscle myosin heavy chain isoform SMemb	S67247	Homo sapiens cDNA: FLJ23324 fis, clone HEP12482, highly similar to HUMMYOHC Human nonmuscle myosin heavy chain-B (MYH10) mRNA	
32755_at	ACTA2	X13839	actin, alpha 2, smooth muscle, aorta	
33994_g_at	MLC	M22919	Human nonmuscle/smooth muscle alkali myosin light chain gene, complete cds.	
33447_at	MLCB	X54304	myosin, light polypeptide, regulatory, non-sarcomeric (20 kD)	
32313_at	TPM2	M12125	tropomyosin 2 (beta)	OMIM Notes: Fibroblast and muscle isoforms result from alternative splicing on exons 6 and 9.
35362_at	MYO10	AB018342	myosin X	
34306_at	MBNL	AB007888	muscleblind-like (Drosophila)	
36989_at	DAG1	L19711	dystroglycan 1 (dystrophin-associated glycoprotein 1)	
40022_at	FCMD	AB008226	Fukuyama type congenital muscular dystrophy (fukutin)	?Walker-Warburg syndrome; Muscular dystrophy, Fukuyama congenital
39031_at	COX7A1	AA152406	cytochrome c oxidase subunit VIIa polypeptide 1 (muscle)	
35729_at	MYO1D	AB018270	myosin 1D	
32378_at	PKM2	M26252	pyruvate kinase, muscle	
40375_at	EGR3	X63741	early growth response 3; OMIM Notes: Expressed in developing muscle spindles.	
1637_at	MAPKAPK3	U09578	mitogen-activated protein kinase-activated protein kinase 3; OMIM Notes: Expressed especially high in heart and skeletal muscle.	
40399_r_at	MEOX2	A1743406	mesenchyme homeobox 2 (growth arrest-specific homeobox). OMIM Notes: Important regulator of myogenesis.	
39565_at	BMPR1A	Z22535	bone morphogenetic protein receptor, type 1A. OMIM Notes: Alternative title, activin receptor-like kinase 3; ALK3. Expressed almost exclusively in skeletal muscle with weak expression in heart and placenta.	Polyposis, juvenile intestinal
41449_at	SGCE	AJ000534	sarcoglycan, epsilon	Dystonia, myoclonic

TABLE 8

<u>Fibroblast (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
39333__at	COL4A1	M26576	Human alpha-1 collagen type IV gene, exon 52.	OMIM Notes: Expressed in fetal normal mesenchymal tissues and stromal fibroblasts within common types of epithelial tumors.
37037__at	P4HA1	M24486	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I	
36666__at	P4HB	M22806	precursor; Human prolyl 4-hydroxylase beta-subunit and disulfide isomerase (P4HB) gene, exon 11, clones 6B-(1,3,5,6).	
41504__s_at	MAF	AF055376	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	
39757__at	SDC2	J04621	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)	
39945__at	FAP	U09278	fibroblast activation protein, alpha	
32835__at	MAFF	AA725102	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	
32535__at	FBN1	X63556	fibrillin 1 (Marfan syndrome)	
2057__g_at	FGFR1	M34641	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	
1380__at	FGF7	M60828	fibroblast growth factor 7 (keratinocyte growth factor)	
32313__at	TPM2	M12125	tropomyosin 2 (beta)	OMIM Notes: Fibroblast and muscle isoforms result from alternative splicing on exons 6 and 9.
31720__s_at	FN1	M10905	fibronectin 1	
31719__at	FN1	X02761	fibronectin 1	
35835__at	PDL-108	AB019409	periodontal ligament fibroblast protein	
34390__at	P4HA2	U90441	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II	

TABLE 9

<u>Adipocyte (Seshi, B, et al)</u>				
Probe ID	Gene Name	Genbank ID	Description	OMIM Notes
34378__at	ADRP	X97324	adipose differentiation-related protein (adipophilin)/lipid-droplet binding/adipocyte-specific	mRNA levels are induced rapidly and maximally after triggering adipocyte differentiation.
40282__s_at	DF	M84526	D component of complement (adipsin)	High level of expression in fat.
33337__at	DEGS	AF002668	degenerative spermatocyte homolog, lipid desaturase ( <i>Drosophila</i> )	
39673__i_at	ECM2	AB011792	extracellular matrix protein 2, female organ and adipocyte specific	
39674__r_at	ECM2	AB011792	extracellular matrix protein 2, female organ and adipocyte specific	
31504__at	HDLBP	M64098	high density lipoprotein binding protein (vigilin)	
37542__at	LHFPL2	D86961	lipoma HMGIC fusion partner-like 2	
36073__at	NDN	U35139	needin homolog (mouse)/Prader-Willi syndrome	

TABLE 9-continued

<u>Adipocyte (Seshi, B, et al)</u>				
Probe ID	Gene Name	Genbank ID	Description	OMIM Notes
37122__at	PLIN	AB005293	Perilipin (Did not meet the criteria to be included in stromal cell gene list because it was positive in 5 of 5 cMPC and 9 of 10 sMPCs, but only 6 of 8 cUSC instead of 7 of 8 cUSC samples).	Plays an important role in adipocyte metabolism. Has significant sequence relationship with ADRP.

TABLE 10

<u>Epithelial cell/carcinoma (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
38590__r_at	PTMA	M14630	prothymosin, alpha (gene sequence 28)	Epidermolytic hyperkeratosis
38589__i_at	PTMA	M14630	prothymosin, alpha (gene sequence 28)	
38610__s_at	KRT10; KPP	X14487	unnamed protein product; Human gene for acidic (type I) cytokeratin 10.	
37326__at	A4	U93305	integral membrane protein; swiss-prot accession: O04901; may play role in cell differentiation in intestinal epithelium	Pancreatic cancer; Polyposis, juvenile intestinal
36812__at	BCAR3	U92715	breast cancer anti-estrogen resistance 3	
36953__at	MADH4	U44378	MAD, mothers against decapentaplegic homolog 4 ( <i>Drosophila</i> )	
36852__at	N33	U42349	Putative prostate cancer tumor suppressor	?Prostate cancer, susceptibility to
36851__g_at	N33	U42360	39 kDa protein; Human N33 protein form 2 (N33) gene, exon 11 and complete cds.	
37762__at	EMP1	Y07909	epithelial membrane protein 1	/receptor
37731__at	EPS15	Z29064	epidermal growth factor receptor pathway substrate 15	
40856__at	SERPINF1; PEDF; EPC-1	U29953	PEDF; Human pigment epithelium-derived factor gene, complete cds.	/associates with p110(RB)
41431__at	ICK	AB023153	intestinal cell kinase	
39363__at	BC-2	AF042384	putative breast adenocarcinoma marker (32 kD)	
39631__at	EMP2	U52100	epithelial membrane protein 2	/associates with p110(RB)
39542__at	ENC1	AF059611	ectodermal-neural cortex (with BTB-like domain)	
40454__at	FAT	X87241	FAT tumor suppressor homolog 1 ( <i>Drosophila</i> )	
32781__f_at	BPAG1	AA058762	bullous pemphigoid antigen 1 (230/240 kD)	Monilethrix /Binds and transports polymeric immunoglobulin
32780__at	BPAG1	AB018271	bullous pemphigoid antigen 1 (230/240 kD)	
32329__at	KRTHB6	X99142	keratin, hair, basic, 6 (monilethrix)	
34005__at	PIGR	X73079	polymeric immunoglobulin receptor, expressed in glomerular epithelial cells.	
1846__at	LGALS8	L78132	lectin, galactoside-binding, soluble, 8 (galectin 8); OMIM Notes: Expressed in prostate carcinoma cells but only rarely in prostatic hypertrophy.	

TABLE 11

<u>Endothelial cell (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
32755__at	ACTA2	X13839	actin, alpha 2, smooth muscle, aorta	/ligand for the TIE2 receptor
39315__at	ANGPT1	D13628	angiopoietin 1	
1929__at	ANGPT1	U83508	angiopoietin 1	
40387__at	EDG2	U80811	endothelial differentiation, lysophosphatidic acid (LPA) G-protein-coupled receptor, 2	

TABLE 11-continued

<u>Endothelial cell (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
40874__at	EDF1	AJ005259	endothelial differentiation-related factor 1; OMIM Notes: EDF1 level inversely correlates with the level of endothelial differentiation. Inhibition of EDF1 expression promotes endothelial cell differentiation. It is postulated that EDF1 may function as a bridging molecule that interconnects regulatory proteins and the basal transcriptional machinery, thus modulating the transcription of the genes involved in endothelial differentiation.	
37907__at	F8A; DXS522E	M34677	Factor VIII-associated gene 1; CpG island protein; Human nested gene protein gene, complete cds.	
41433__at	VCAM1	M73255	Human vascular cell adhesion molecule-1 (VCAM1) gene, complete CDS.	
36988__at	TNFAIP1	M80783	tumor necrosis factor, alpha-induced protein 1 (endothelial); OMIM Notes: Involved in the primary response of the endothelium to TNF.	
583__s_at	VCAM1	M30257	vascular cell adhesion molecule 1	
1953__at	VEGF	AF024710	vascular endothelial growth factor	
36100__at	VEGF	AF022375	vascular endothelial growth factor	
37268__at	VEGFB	U43368	vascular endothelial growth factor B	
159__at	VEGFC	U43142	vascular endothelial growth factor C	/ligand and activator of the receptor tyrosine kinase Flt4

TABLE 12

<u>Nerve cell/neuroendocrine/neurologic disorders (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
37298__at	GABARAP	AF044671	GABA(A) receptor-associated protein	
37692__at	DBI	AI557240	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	
35767__at	GABARAPL2	AI565760	GABA(A) receptor-associated protein-like 2	
35785__at	GABARAPL1	W28281	GABA(A) receptor-associated protein like 1	
38406__f_at	PTGDS	AI207842	prostaglandin D2 synthase (21 kD, brain)	
38657__s_at	CLTA	M20471	clathrin, light polypeptide (Lca), brain-specific insertion sequences	
38653__at	PMP22	D11428	peripheral myelin protein 22	Charcot-Marie-Tooth disease with deafness; Charcot-Marie-Tooth neuropathy-1A; Dejerine-Sottas disease; Neuropathy, recurrent, with pressure palsies
38291__at	PENK	J00123	preproenkephalin (; Human enkephalin gene: exon 3 and 3'flank.	
39072__at	MXI1	L07648	MAX interacting protein 1	Neurofibrosarcoma; Prostate cancer, susceptibility to/ transcription factor; forms heterodimers with Max protein
38841__at	GDBR1	AF068195	putative glioblastoma cell differentiation-related	
38818__at	SPTLC1	Y08685	serine palmitoyltransferase, long chain base subunit 1	Neuropathy, hereditary sensory and autonomic, type 1
36990__at	UCHL1	X04741	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase), neuron-	Parkinson disease, familial

TABLE 12-continued

Nerve cell/neuroendocrine/neurologic disorders (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
37005_at	NBL1	D28124	specific. OMIM Notes: Highly specific to neurons and to cells of the diffuse neuroendocrine system and their tumors.	/peripheral proteins believed to act as membrane-cytoskeleton linker molecules
37286_at	NRCAM	AB002341	neuroblastoma, suppression of tumorigenicity 1	
36667_at	PYGB	U47025	neuronal cell adhesion molecule phosphorylase, glycogen; brain	
36965_at	ANK3	U13616	ankyrin 3, node of Ranvier (ankyrin G)	
38040_at	SPF30	AF107463	splicing factor 30, survival of motor neuron-related	
37958_at	BCMP1	AL049257	brain cell membrane protein 1	
41221_at	PGAM1	J04173	phosphoglycerate mutase 1 (brain)	
40936_at	CRIM1	AI651806	cysteine-rich motor neuron 1	
41091_at	FALZ	U05237	fetal Alzheimer antigen. OMIM Notes: Abnormally expressed in fetal brain. The corresponding antibody ALZ50 recognizes neurofibrillary pathology associated with Alzheimer's disease.	Alzheimer disease-1, APP-related; Amyloidosis, cerebroarterial, Dutch type; Schizophrenia, chronic
41136_s_at	APP	Y00264	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)	
763_at	GMFB	AB001106	glia maturation factor, beta	
641_at	PSEN1	L76517	presenilin 1 (Alzheimer disease 3)	
39793_at	GBAS	AF029786	glioblastoma amplified sequence	/associates with p110(RB) OMIM Notes: Expressed highest in brain.
40023_at	BDNF	X60201	brain-derived neurotrophic factor	
39687_at	E46L	AI524873	like mouse brain protein E46	
39686_g_at	E46L	AL050282	like mouse brain protein E46	
39542_at	ENC1	AF059611	ectodermal-neural cortex (with BTB-like domain)	Parangliomas, familial central nervous system; Parangliomas, familial nonchromaffin, 1, with and without deafness; Pheochromocytoma
40193_at	ENO2	X51956	Human ENO2 gene for neuron specific (gamma) enolase.	
40121_at	HIP2	U58522	huntingtin interacting protein 2	
40467_at	SDHD	AB006202	succinate dehydrogenase complex, subunit D, integral membrane protein	
40281_at	NEDD5	D63878	neural precursor cell expressed, developmentally down-regulated 5	Ceroid-lipofuscinosis, neuronal 2, classic late infantile
32824_at	CLN2	AF039704	deficient in late-infantile neuronal ceroid lipofuscinosis; <i>Homo sapiens</i> lysosomal pepstatin insensitive protease (CLN2) gene, complete cds.	
32607_at	BASP1	AF039656	brain abundant, membrane attached signal protein 1	
33817_at	D10S102	S63912	FBRNP; heterogeneous ribonucleoprotein homolog; This sequence comes from FIG. 3; D10S102 = FBRNP [human, fetal brain, mRNA, 3043 nt].	
33942_s_at	STXBP1	AF004563	syntaxin binding protein 1	/implicated in vesicle trafficking and neurotransmitter release
1659_s_at	RHEB2	D78132	Ras homolog enriched in brain 2	
1695_at	NEDD8	D23662	neural precursor cell expressed, developmentally down-regulated 8	
2053_at	CDH2	M34064	cadherin 2, type 1, N-cadherin (neuronal)	
216_at	PTGDS	M98539	Human prostaglandin D2 synthase gene, exon 7, brain	Spastic ataxia, Charlevoix-Saguenay type
32102_at	SACS	AB018273	spastic ataxia of Charlevoix-Saguenay (sacsin)	
31896_at	NAG	AL050281	neuroblastoma-amplified protein	

TABLE 12-continued

Nerve cell/neuroendocrine/neurologic disorders (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
35681_r_at	ZFHX1B	AB011141	zinc finger homeobox 1b. OMIM Notes: SMAD-interacting protein 1 (SMADIP1) appears to be essential to embryonic neural and neural crest development.	Hirschsprung disease-mental retardation syndrome; Hirschsprung disease-mental retardation syndrome without Hirschsprung disease
35268_at	AXOT	AL050171	axotrophin	
36190_at	CDR2	M63256	cerebellar degeneration-related protein (62 kD)	
36609_at	SLC1A3	D26443	solute carrier family 1 (glial high affinity glutamate transporter), member 3	
35973_at	HYPH	AB023163	Huntingtin interacting protein H	
36142_at	SCA1	X79204	spinocerebellar ataxia 1 (olivopontocerebellar ataxia 1, autosomal dominant, ataxin 1)	Spinocerebellar ataxia-1
34817_s_at	A2LP	U70671	ataxin 2 related protein	
34777_at	ADM	D14874	adrenomedullin	
34394_at	ADNP	AB018327	activity-dependent neuroprotector	
32606_at	BASP1	AA135683	brain abundant, membrane attached signal protein 1	
38233_at	HOMER-3	AF093265	Homer, neuronal immediate early gene, 3	
36998_s_at	SCA2	Y08262	spinocerebellar ataxia 2 (olivopontocerebellar ataxia 2, autosomal dominant, ataxin 2)	Spinocerebellar ataxia-2
35150_at	TNFRSF5	X60592	tumor necrosis factor receptor superfamily, member 5	Immunodeficiency with hyper-IgM, type 3
34166_at	SLC6A7	S80071	solute carrier family 6 (neurotransmitter transporter, L-proline), member 7	
34265_at	SGNE1	Y00757	secretory granule, neuroendocrine protein 1 (7B2 protein)	
654_at	MXI1	L07648	MAX interacting protein 1	Neurofibrosarcoma; Prostate cancer, susceptibility to/ transcription factor; forms heterodimers with Max protein
37945_at	BACH	U91316	brain acyl-CoA hydrolase	
39685_at	E46L	AL050282	like mouse brain protein E46	
33769_at	MPZL1	AF087020	myelin protein zero-like 1	
39356_at	NEDD4L	AB007899	neural precursor cell expressed, developmentally down-regulated 4-like	
38800_at	STMN2	D45352	stathmin-like 2; OMIM Notes: Neuronal growth-associated protein SCG10.	
36933_at	NDRG1	D87953	N-myc downstream regulated gene 1	Neuropathy, hereditary motor and sensory, Lom type
40140_at	ZFP103	D76444	zinc finger protein 103 homolog (mouse); OMIM Notes: Alternative title, KF1, expressed in normal cerebellum and Alzheimer disease cerebral cortex, but not in normal cerebral cortex.	
1452_at	LMO4	U24576	LIM domain only 4	OMIM Notes: is highly expressed in the cranial neural crest cells, somite, dorsal limb bud mesenchyme, motor neurons, Schwann cell progenitors, and T-lymphocyte lineage.
1058_at	WASF3	S69790	WAS protein family, member 3	

TABLE 13

<u><i>Drosophila</i> and/or homeotic genes (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
38288__at	SNAI2	U69196	snail homolog 2 ( <i>Drosophila</i> ). OMIM Notes: Neural crest transcription factor SLUG. A zinc finger protein that plays an important role in the transition of epithelial to mesenchymal characteristics within the neural crest.	
39037__at	MLLT2	L13773	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, <i>Drosophila</i> ); translocated to, 2	
39070__at	SNL	U03057	singed-like (fascin homolog, sea urchin) ( <i>Drosophila</i> ). OMIM Notes: Positive in dendritic cells of lymph nodes and Reed-Sternberg cells.	
39164__at	ARIH2	AF099149	ariadne homolog 2 ( <i>Drosophila</i> ). OMIM Notes: Upregulated during retinoic acid-induced granulocytic differentiation of APL cells.	
38750__at	NOTCH3	U97669	Notch homolog 3 ( <i>Drosophila</i> ). OMIM Notes: Promotes the differentiation of astroglia from multipotent progenitors.	Cerebral (autosomal dominant) arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).
38944__at	MADH3	U68019	MAD, mothers against decapentaplegic homolog 3 ( <i>Drosophila</i> )	OMIM Notes: SMAD3 signal transduction important in the regulation of muscle-specific genes.
37693__at	NUMB	L40393	numb homolog ( <i>Drosophila</i> )	OMIM Notes: Numb directs neuronal cell fate decisions.
40004__at	SIX1	X91868	sine oculis homeobox homolog 1 ( <i>Drosophila</i> )	OMIM Notes: Expressed in adult skeletal muscle, and in multiple tumors including mammary carcinoma.
39610__at	HOXB2	X16665	homeo box B2	OMIM Notes: Essential for motor neuron development. Within the hematopoietic compartment, expressed specifically in erythromegakaryocytic cell lines.
40575__at	DLG5	AB011155	discs, large ( <i>Drosophila</i> ) homolog 5	OMIM Notes: Expressed in prostate gland epithelial cells.
40570__at	FOXO1A	AF032885	forkhead box O1A (rhabdomyosarcoma)	Rhabdomyosarcoma, alveolar. OMIM Notes: Activates myogenic transcription program.
40127__at	PMX1	M95929	paired mesoderm homeo box 1	OMIM Notes: Expressed in cardiac, skeletal and smooth muscle tissues.
40454__at	FAT	X87241	FAT tumor suppressor homolog 1 ( <i>Drosophila</i> )	OMIM Notes: Expressed in many epithelial, some endothelial and smooth muscle cells.
40328__at	TWIST	X99268	twist homolog (acrocephalosyndactyly 3; Saethre-Chotzen syndrome) ( <i>Drosophila</i> )	Saethre-Chotzen syndrome. OMIM Notes: Required for cranial neural tube morphogenesis.
33222__at	FZD7	AB017365	frizzled homolog 7 ( <i>Drosophila</i> )	OMIM Notes: Highest expression adult skeletal muscle and fetal kidney. FZD7 dependent PKC signaling controls cell sorting behaviour in the mesoderm.
32696__at	PBX3	X59841	pre-B-cell leukemia transcription factor 3	
33337__at	DEGS	AF002668	degenerative spermatocyte homolog, lipid desaturase ( <i>Drosophila</i> ); adipocyte associated.	
1857__at	MADH7	AF010193	MAD, mothers against decapentaplegic homolog 7	OMIM Notes: MAD proteins were originally

TABLE 13-continued

<u><i>Drosophila</i> and/or homeotic genes (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
			( <i>Drosophila</i> )	defined in <i>Drosophila</i> as essential components of the signaling pathways of the TGF-beta receptor family (e.g., TGFBR1). MADH7 and MADH6 as shown by IHC and ISH are predominantly expressed in vascular endothelium. /inhibitor of BMP signaling
1955_s_at	MADH6	AF035528	MAD, mothers against decapentaplegic homolog 6 ( <i>Drosophila</i> )	
1013_at	MADH5	U59913	MAD, mothers against decapentaplegic homolog 5 ( <i>Drosophila</i> )	OMIM Notes: SMAD5 plays a critical role in the signaling pathway by which TGF-beta inhibits the proliferation of human hematopoietic progenitor cells.
1453_at	MADH2	U68018	MAD, mothers against decapentaplegic homolog 2 ( <i>Drosophila</i> )	
1433_g_at	MADH3	U68019	MAD, mothers against decapentaplegic homolog 3 ( <i>Drosophila</i> )	OMIM Notes: SMAD2/SMAD3 signal transduction appears to be important in the regulation of muscle-specific genes.
35681_r_at	ZFHX1B	AB011141	zinc finger homeobox 1b. OMIM Notes: SMAD-interacting protein 1 (SMADIP1) appears to be essential to embryonic neural and neural crest development.	Hirschsprung disease-mental retardation syndrome; Hirschsprung disease-mental retardation syndrome without Hirschsprung disease
35226_at	EYA2	U71207	eyes absent homolog 2 ( <i>Drosophila</i> )	OMIM Notes: Expressed in extensor tendons, and in lens fibers and participates in connective tissue patterning.
36308_at	ZIC1	D76435	Zic family member 1 (odd-paired homolog, <i>Drosophila</i> )	OMIM Notes: Specifically expressed in nervous tissue and in particular cerebellar granule cells, potential biomarker for cerebellar granule cell lineage and medulloblastoma.
34306_at	MBNL	AB007888	muscleblind-like ( <i>Drosophila</i> )	OMIM Notes: Expressed in skeletal muscle myoblasts, also in lymphoblastoid cell lines.
33710_at	C3F	U72515	putative protein similar to nessy ( <i>Drosophila</i> )	OMIM Notes: Expressed in fibroblasts and hepatocytes.

TABLE 14

<u>B-cell/B-cell neoplasms (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
41562_at	BMI1	L13689	B lymphoma Mo-MLV insertion region (mouse)	/proto-oncogene
37294_at	BTG1	X61123	B-cell translocation gene 1, anti-proliferative	Centrocytic lymphoma; Leukemia/lymphoma, B-cell, 1; Multiple myeloma; Parathyroid adenomatosis 1
38418_at	CCND1	X59798	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	
37730_at	p100	U22055	EBNA-2 co-activator (100 kD); OMIM Notes: EBNA-2 activates transcription of specific genes and is essential for EBV-mediated B-lymphocyte transformation.	/associates with the EBV nuclear protein 2 acidic domain



TABLE 14-continued

B-cell/B-cell neoplasms (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
466__at	GTF2I	U77948	general transcription factor II, I; OMIM Notes: Alternative title, BTK-associated protein, 135 kD (BAP135). Bruton's tyrosine kinase (BTK) is essential for B-cell activation and phosphorylates BAP135 in B cells.	Leukemia, chronic myeloid
36875__at	IBTK	AL050018	inhibitor of Bruton's tyrosine kinase	
38438__at	NFKB1	M58603	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	
39730__at	ABL1	X16416	v-abl Abelson murine leukemia viral oncogene homolog 1	
38743__f_at	RAF1	X06409	v-raf-1 murine leukemia viral oncogene homolog 1	
36645__at	RELA	L19067	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	Stem-cell leukemia/lymphoma syndrome
41436__at	ZNF198	AJ224901	zinc finger protein 198; OMIM Notes: ZNF198 involves T- or B-cell lymphoblastic lymphoma, myeloid hyperplasia, and eosinophilia and evolves toward AML. This multilineage involvement suggests the malignant transformation of primitive hematopoietic stem cell.	
40091__at	BCL6	U00115	B-cell CLL/lymphoma 6 (zinc finger protein 51); OMIM Notes: BCL6 is predominantly expressed in the B-cell lineage, especially mature B cells (centrocytes and centroblasts).	Lymphoma, B-cell, Diffuse Large
32776__at	RALB	M35416	v-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	/Binds and transports polymeric immunoglobulin
32696__at	PBX3	X59841	pre-B-cell leukemia transcription factor 3	
33791__at	DLEU1	Y15227	deleted in lymphocytic leukemia, 1	
34005__at	PIGR	X73079	polymeric immunoglobulin receptor	
1636__g_at	ABL	U07563	ABL is the cellular homolog proto-oncogene of Abelson's murine leukemia virus and is associated with the t(9;22) chromosomal translocation with the BCR gene in chronic myelogenous and acute lymphoblastic leukemia.	Leukemia, chronic myeloid
1728__at	BMI1	L13689	B lymphoma Mo-MLV insertion region (mouse)	/proto-oncogene
2020__at	CCND1	M73554	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	Centrocytic lymphoma; Leukemia/lymphoma, B-cell, 1; Multiple myeloma; Parathyroid adenomatosis 1
1295__at	RELA	L19067	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	/IkB-like activity
1377__at	NFKB1	M58603	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	
1461__at	NFKBIA	M69043	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	
1389__at	MME	J03779	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	
35350__at	GALNAC4 S-6ST	AB011170	B cell RAG associated protein	/basic helix-loop-helix transcription factor
35992__at	MSC	AF087036	musculin (activated B-cell factor-1, ABF1); OMIM Notes: Downstream target of B-cell receptor signal transduction pathway. Also expressed in proliferating undifferentiated myeloblasts.	
34344__at	IKBKAP	AF044195	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	Dysautonomia, familial
34350__at	RSN	X64838	restin (Reed-Sternberg cell-expressed intermediate filament-associated protein); Note R-S cell is a form of B-cell.	
36204__at	PTPRF	Y00815	protein tyrosine phosphatase, receptor type, F; OMIM Notes: Alternative title, Leukocyte	

TABLE 14-continued

<u>B-cell/B-cell neoplasms (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
34391__at	IGBP1	Y08915	antigen-related tyrosine phosphatase (LAR). Both LAR and LCA (CD45) map to chromosome 1. LCA is protein-tyrosine phosphatase, receptor-type C, PTPRC, whereas LAR is PTPRF. immunoglobulin (CD79A) binding protein 1.	IGBP1, a marker for early B-cells
1373__at	TCF3	M31523	proto-oncogene or transcription factor 3 TCF3 (E2A immunoglobulin enhancer binding factors E12/E47); OMIM Notes: E2A mutant mice will have selective failure to develop B cells, all other hematopoietic cell lineages being intact. The block to B cell development occurs before immunoglobulin D(H)-J(H) rearrangement.	Leukemia, acute lymphoblastic. A homeobox gene contributing the DNA binding domain of the t(1;19) translocation protein in precursor B-cell ALL.
35150__at	TNFRSF5	X60592	tumor necrosis factor receptor superfamily, member 5; OMIM notes: Alternative title, B-cell associated molecule CD40; expressed on the surface of all mature B cells, most mature B-cell malignancies and some early B-cell ALL.	Immunodeficiency with hyper-IgM, type 3
38740__at	ZFP36L1	X79067	zinc finger protein 36, C3H type-like 1; OMIM Notes: Alternative title, BERG36 (B-cell early response gene encoding a 36 kD protein).	
37026__at	COPEB	AF001461	core promoter element binding protein; OMIM Notes: Alternative title, B-cell-derived 1, BCD1. The expression of BCD1 was limited to two tissues, CD19+ B-cells and testis of normal individuals. B-cell maturation is associated with BCD1 expression.	/transcription factor involved in hepatic wound healing
38050__at	BTF	D79986	Bcl-2-associated transcription factor	
32696__at	PBX3	X59841	pre-B-cell leukemia transcription factor 3	

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TABLE 15

<u>Myeloid cell/myeloid leukemia (Seshi, B)</u>				
Systematic	Common	Genbank	Description	Phenotype/Function
39037__at	MLLT2	L13773	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, <i>Drosophila</i> ); translocated to, 2	
37486__f_at	MEIS3	U68385	Meis1, myeloid ecotropic viral integration site 1 homolog 3 (mouse)	
37685__at	PICALM	U45976	phosphatidylinositol binding clathrin assembly protein	Leukemia, acute myeloid; Leukemia, acute T-cell lymphoblastic
41220__at	MSF	AB023208	MLL septin-like fusion; a fusion partner gene of MLL	Leukemia, acute myeloid, therapy-related; Ovarian carcinoma
41175__at	CBFB	L20298	core-binding factor, beta subunit	Myeloid leukemia, acute, M4Eo subtype
943__at	RUNX1	D43968	nunt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	Leukemia, acute myeloid; Platelet disorder, familial, with associated myeloid malignancy
39730__at	ABL1	X16416	v-abl Abelson murine leukemia viral oncogene homolog 1	Leukemia, chronic myeloid
33146__at	MCL1	L08246	myeloid cell leukemia sequence 1 (BCL2-related)	
1636__g_at	ABL	U07563	ABL is the cellular homolog proto-oncogene of Abelson's murine leukemia virus and is associated with the t9;22 chromosomal translocation with the BCR gene in chronic myelogenous and acute lymphoblastic leukemia.	Leukemia, chronic myeloid

TABLE 15-continued

Myeloid cell/myeloid leukemia (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
277__at	MCL1	L08246	myeloid cell leukemia sequence 1 (BCL2-related)	
41388__at	MEIS2	AF017418	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	
40189__at	SET	M93651	SET translocation (myeloid leukemia-associated, M2/M4 AML); SET stands for suppressor of variegation, enhancer of zeste and trithorax.	
38992__at	DEK	X64229	DEK oncogene (DNA binding)	Leukemia, acute nonlymphocytic
36941__at	AF1Q	U16954	ALL1-fused gene from chromosome 1q	Leukemia, acute myelomonocytic

TABLE 16

T cell/NK cell (Seshi, B)				
Systematic	Common	Genbank	Description	Phenotype/Function
37685__at	PICALM	U45976	phosphatidylinositol binding clathrin assembly protein	Leukemia, acute myeloid; Leukemia, acute T-cell lymphoblastic
498__at	TAX1BP1	U33821	Tax1 (human T-cell leukemia virus type I) binding protein 1	
40822__at	NFATC3	L41067	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	
34003__at	CD4	U47924	major receptor for HIV-1; member of immunoglobulin supergene family; T cell surface glycoprotein T4	/T-cell coreceptor; involved in antigen recognition; participant in signal transduction pathway
32602__at	RAP1GDS1	X63465	RAP1, GTP-GDP dissociation stimulator 1	Lymphocytic leukemia, acute T-cell (T-ALL)
35279__at	TAX1BP1	U33821	Tax1 (human T-cell leukemia virus type I) binding protein 1	
34234__f_at	NKTR	AI688640	natural killer-tumor recognition sequence; OMIM Notes: The protein product of the NKTR gene is present on the surface of LGLs and facilitates their binding to tumor targets.	
39426__at	TCERG1	AF017789	transcription elongation regulator 1 (CA150)	/HIV-1 Tat transcriptional coactivator
32602__at	RAP1GDS1	X63465	RAP1, GTP-GDP dissociation stimulator 1	Lymphocytic leukemia, acute T-cell

TABLE 17

Stromal cells showing expression of genes that are typically affiliated with B-cell progenitors.					
Gene name	Probe ID	Genbank ID	cUSC	cMPC	sMPC
CD45	40518__at	Y00062	Positive in 8/8 samples	Positive in 4/5 samples	Positive in 6/10 samples
CD34	538__at	S53911	5/8	4/5	4/10
CD19	1116__at	M28170	0/8	0/5	10/10
CD20	619__s_at	M27394	0/8	1/5	3/10
CD22	38521__at	X59350	2/8	0/5	1/10
*CD10 (CALLA)	1389__at	J03779	8/8	5/5	10/10
*TCF3 (E2A)	1373__at	M31523	8/8	5/5	9/10
*CD79A (IGBP1)	34391__at	Y08915	8/8	5/5	9/10
*HLA class II, Dr alpha	37039__at	J00194	8/8	5/5	9/10
*HLA class II, Dr beta 1	33261__at	M16941	8/8	5/5	10/10
*B2 microglobulin	34644__at	AB021288	8/8	5/5	10/10
*BMI1	41562__at	L13689	8/8	5/5	10/10
CD2	40738__at	M16336	2/8	1/5	2/10

TABLE 17-continued

Stromal cells showing expression of genes that are typically affiliated with B-cell progenitors.					
Gene name	Probe ID	Genbank ID	cUSC	cMPC	sMPC
CD3 epsilon	36277__at	M23323	4/8	3/5	3/10
CD5	32953__at	X04391	0/8	0/5	0/10
CD7	771__s__at	D00749	0/8	0/5	0/10
CD13	39385__at	M22324	8/8	5/5	0/10
CD33	36802__at	M23197	4/8	2/5	0/10
CD14	36661__s__at	X06882	8/8	3/5	0/10

Footnote to Table 17

Genes marked with asterisk (\*) met the criteria for inclusion in stromal cell gene list.

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TABLE 18

Stromal cell gene lists associated with diverse cellular lineages.	
Cell lineage	Representative examples of associated genes
A) Osteoblast (Table 6)	Cadherin 11 (type 2, OB-cadherin, osteoblast), osteonectin, osteopontin, osteoblast specific factor 2 (fasciclin I-like), chondroitin sulfate proteoglycan 2 (versican), biglycan, barnacan, collagen, type I, alpha 2 (Osteogenesis imperfecta)
B) Muscle (Table 7)	Various types of myosin, tropomyosin 1 and 2, transgelin, transgelin 2, caldesmon 1, dystrophin, dystroglycan 1, Fukuyama type congenital muscular dystrophy (fukutin), ATPase (Ca <sup>++</sup> transporting, cardiac muscle, slow twitch 2/Darier disease), capping protein (actin filament) muscle Z-line (alpha 2 and beta)
C) Fibroblast (Table 8)	Prolyl 4-hydroxylase, fibronectin, fibrillin 1, fibroglycan, alpha-1 collagen type IV gene, fibroblast growth factor 7 (keratinocyte growth factor) and periodontal ligament fibroblast protein
D) Adipocyte (Table 9)	Adipose differentiation-related protein (adipophilin), adipsin, lipid desaturase, ECM protein 2 (adipocyte specific), vigilin, needin and perilipin
E) Epithelial cell/carcinoma (Table 10)	Cytokeratin 10, keratin (hair, basic, 6, monilethrix), epithelial membrane protein 1, epithelial membrane protein 2, bullous pemphigoid antigen 1, milk fat globule-EGF factor 8 protein (lactadherin), breast epithelial mucin-associated antigen, prothymosin alpha and thymosin beta 4 and beta 10
F) Endothelial cell/angiogenesis/vasculogenesis (Table 11 and Footnote 1)	Angiopoietin, VEGF, VCAM1, Factor VIII-associated gene, EDF1 (endothelial differentiation-related factor 1) and EDG2 (endothelial differentiation, G-protein-coupled receptor, 2)
G) Neural cell (Table 12)	Neuron-specific (gamma) enolase, GABA receptor-associated proteins, NCAM, N-cadherin, presenilin 1, Huntingtin-interacting protein, adrenomedullin, axotrophin, brain-derived neurotrophic factor (BDNF), syntaxin binding protein 1, peripheral myelin protein 22, ankyrin 3 (node of Ranvier, ankyrin G), glial maturation factor, beta
H) Myeloid cell/myeloid leukemia (Table 15 and	MLLT2 (mixed-lineage leukemia (trithorax homolog, <i>Drosophila</i> ), CBFb (core-binding factor, beta subunit), ABL proto-oncogene, MCL1 (myeloid cell leukemia sequence 1) and

TABLE 18-continued

Stromal cell gene lists associated with diverse cellular lineages.	
Cell lineage	Representative examples of associated genes
Footnote 2)	DEK oncogene
I) T cell/NK cell/leukemia (Table 16 and Footnote 3)	CD4, TAX1 binding protein 1, natural killer-tumor recognition sequence, RAP1, GTP-GDP association stimulator 1
J) B-cell/B-cell neoplasms (Table 14)	Bruton's tyrosine kinase-associated protein, 135 kD (BAP135), inhibitor of Bruton's tyrosine kinase, pre-B-cell leukemia transcription factor 3 (PBX3), B cell RAG associated protein, cyclin D1, BCL6, TCF 3 (E12/E47), CALLA (CD10), CD79A, COPEB (core promoter element binding protein, expression limited to CD19+ B cells and testis), protein tyrosine phosphatase, receptor type, F (similar to CD45, which is PTPRC), restin (expressed in Reed-Sternberg cells in Hodgkin's lymphoma, known as a type of B-cell lymphoma)

TABLE 19

Human homologs of <i>Drosophila</i> genes, representing diverse cellular pathways, are simultaneously active in a stromal cell.			
Representative examples of associated genes			
Cell lineage	Gene	Brief description	
Neural	SNAI2	snail homolog 2 ( <i>Drosophila</i> ).	
	NOTCH3	Notch homolog 3 ( <i>Drosophila</i> ).	
	NUMB	numb homolog ( <i>Drosophila</i> )	
	HOXB2	homeo box B2	
Muscle	TWIST	twist homolog (acrocephalosyndactyly 3) ( <i>Drosophila</i> )	
	ZIC1	Zic family member 1 (odd-paired homolog, <i>Drosophila</i> )	
	ZFHX1B	zinc finger homeobox 1b.	
	MADH3	MAD, mothers against decapentaplegic homolog 3 ( <i>Drosophila</i> )	
	SIX1	sine oculis homeobox homolog 1 ( <i>Drosophila</i> )	

TABLE 19-continued

Human homologs of <i>Drosophila</i> genes, representing diverse cellular pathways, are simultaneously active in a stromal cell.		
Representative examples of associated genes		
Cell lineage	Gene	Brief description
Adipocyte	FOXO1A	forkhead box O1A (rhabdomyosarcoma)
	PMX1	paired mesoderm homeo box 1
	FZD7	frizzled homolog 7 ( <i>Drosophila</i> )
	MBNL	muscleblind-like ( <i>Drosophila</i> )
	MEOX2	mesenchyme homeobox 2. Important regulator of myogenesis.
	DEGS	degenerative spermatocyte homolog, lipid desaturase ( <i>Drosophila</i> ); adipocyte associated.
	DLG5	discs, large ( <i>Drosophila</i> ) homolog 5
	MADH7	MAD, mothers against decapentaplegic homolog 7 ( <i>Drosophila</i> )
	EYA2	eyes absent homolog 2 ( <i>Drosophila</i> )
	C3F	putative protein similar to nussy ( <i>Drosophila</i> )
Hematopoietic	MLLT2	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, <i>Drosophila</i> ); translocated to, 2
	SNL	singed-like (fascin homolog, sea urchin) ( <i>Drosophila</i> ).
	ARIH2	ariadne homolog 2 ( <i>Drosophila</i> ).
	PBX3	pre-B-cell leukemia transcription factor 3
	MADH5	MAD, mothers against decapentaplegic homolog 5 ( <i>Drosophila</i> )

TABLE 21

Stromal-derived factor (SDF) genes active in a stromal cell			
Probe ID	Gene name	Genbank ID	Brief description
40957_at	JJAZ1	D63881	joined to JAZF1; Endometrial stromal tumors. OMIM Excerpts: JAZF1/JJAZ1 fusion protein present in all types of endometrial tumors.
41627_at	SDF2	D50645	stromal cell-derived factor 2
32666_at	SDF1	U19495	stromal cell-derived factor 1; AIDS, resistance to. OMIM Excerpts: SDF1 inhibits HIV-1 replication.
33834_at	SDF1	L36033	stromal cell-derived factor 1. AIDS, resistance to
35747_at	SDFR1	AF035287	stromal cell derived factor receptor 1
20	Reference to OMIM: Online Mendelian Inheritance in Man, OMIM (TM). McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2000. World Wide Web URL: <a href="http://www.ncbi.nlm.nih.gov/omim/">http://www.ncbi.nlm.nih.gov/omim/</a>		

TABLE 22A

List of genes from Table 3 with Affy data & group statistics		
Gene name	Probe ID	Descriptions
CD45	40518_at	Cluster Incl. Y00062: Human mRNA for T200 leukocyte common antigen (CD45, L.C-A)/cds = (146,3577)/gb = Y00062/gi = 34275/ug = Hs.170121/len = 4597
CD34	538_at	S53911/FEATURE = /DEFINITION = S53911 CD34 = glycoprotein expressed in lymphohematopoietic
30		
35		

TABLE 20

Affymetrix (hybridization and housekeeping) positive-control genes			
Probe ID	Gene Name	Genbank ID	ID Brief description
AFFX-HSAC07/X00351_3_st	ACTB	X00351	actin, beta
AFFX-HSAC07/X00351_3_at	ACTB	X00351	actin, beta
AFFX-BioC-3_at	bioA	J04423	ORF 1
AFFX-BioB-M_at	bioA	J04423	ORF 1
AFFX-BioDn-5_at	bioA	J04423	ORF 1
AFFX-BioDn-3_at	bioA	J04423	ORF 1
AFFX-BioC-5_at	bioA	J04423	ORF 1
AFFX-HUMGAPDH/M33197_3_at	GAPD	M33197	glyceraldehyde-3-phosphate dehydrogenase
AFFX-HUMGAPDH/M33197_5_at	GAPD	M33197	glyceraldehyde-3-phosphate dehydrogenase
AFFX-HUMISGF3A/M97935_3_at	STAT1	M97935	signal transducer and activator of transcription 1, 91 kD/Mycobacterial infection, atypical, familial disseminated
AFFX-CreX-5_at		X03453	pot. ORF1 (aa 1-73); ORF2, put. cre protein (aa 1-343); Bacteriophage P1 cre gene for recombinase protein.
AFFX-CreX-3_at		X03453	pot. ORF1 (aa 1-73); ORF2, put. cre protein (aa 1-343); Bacteriophage P1 cre gene for recombinase protein.
AFFX-hum_alu_at		U14573	Human Alu-Sq subfamily consensus sequence.

TABLE 22A-continued

List of genes from Table 3 with Affy data & group statistics		
Gene name	Probe ID	Descriptions
		progenitor cells {alternatively spliced, truncated form} [human, UT7, mRNA, 2657 nt]
CD19	1116_at	M28170/FEATURE = /DEFINITION = HUMCSPC Human cell surface protein CD19 (CD19) gene, complete cds
CD20	619_s_at	M27394/FEATURE = cds/DEFINITION = HUMB1LYM Human B-lymphocyte cell-surface antigen B1 (CD20)
CD22	38521_at	Cluster Incl. X59350: <i>H. sapiens</i> mRNA for B cell membrane protein CD22/cds = (56,2599)/gb = X59350/gi = 36090/ug = Hs.171763/len = 3250
*CD10 (CALLA)	1389_at	J03779/FEATURE = mRNA/DEFINITION = HUMCALLA Human common acute lymphoblastic leukemia antigen (CALLA) mRNA, complete cds
*TCF3 (E2A)	1373_at	M31523/FEATURE = /DEFINITION = HUMTEFAA Human transcription factor (E2A) mRNA, complete cds
*CD79A (IGBP1)	34391_at	Cluster Incl. Y08915: <i>H. sapiens</i> mRNA for alpha 4 protein/cds = (8,1027)/gb = Y08915/gi = 1877201/ug = Hs.3631/len = 1321
*HLA class II, Dr alpha	37039_at	Cluster Incl. J00194: human hla-dr antigen alpha-chain mrna & ivs fragments/cds = (26,790)/gb = J00194/gi = 188231/ug = Hs.76807/len = 1199
*HLA class II, Dr beta 1	33261_at	Cluster Incl. M16941: Human MHC class II HLA-DR7-associated glycoprotein beta- chain mRNA, complete cds/cds = (23,823)/gb = M16941/gi = 188257/ug = Hs.180255/len = 1146
*B2 micro-globulin	34644_at	Cluster Incl. AB021288: <i>Homo sapiens</i> mRNA for beta 2-microglobulin, complete cds/cds = (13,372)/gb =

TABLE 22A-continued

List of genes from Table 3 with Affy data & group statistics		
Gene name	Probe ID	Descriptions
		AB021288/gi = 4038732/ug = Hs.75415/len = 925
*BMI1	41562_at	Cluster Incl. L13689: Human prot-oncogene (BMI-1) mRNA, complete cds/cds = (479,1459)/gb = L13689/gi = 291872/ug = Hs.431/len = 3203
CD2	40738_at	Cluster Incl. M16336: Human T-cell surface antigen CD2 (T11) mRNA, complete cds, clone PB1/cds = (23,1105)/gb = M16336/gi = 180093/ug = Hs.89476/len = 1522
CD3 epsilon	36277_at	Cluster Incl. M23323: Human membrane protein (CD3-epsilon) gene/cds = (59,682)/gb = M23323/gi = 515731/ug = Hs.3003/len = 1320
CD5	32953_at	Cluster Incl. X04391: Human mRNA for lymphocyte glycoprotein T1/Leu-1/cds = (72,1559)/gb = X04391/gi = 37186/ug = Hs.234745/len = 2320
CD7	771_s_at	D00749/FEATURE = cds/DEFINITION = HUMCD7G3 Human T cell surface antigen CD7 gene, exon 4
CD13	39385_at	Cluster Incl. M22324: Human aminopeptidase N/CD13 mRNA encoding aminopeptidase N, complete cds/cds = (120,3023)/gb = M22324/gi = 178535/ug = Hs.1239/len = 3477
CD33	36802_at	Cluster Incl. M23197: Human differentiation antigen (CD33) mRNA, complete cds/cds = (12,1106)/gb = M23197/gi = 180097/ug = Hs.83731/len = 1437
CD14	36661_s_at	Cluster Incl. X06882: Human gene for CD14 differentiation antigen/cds = (105,1232)/gb = X06882/gi = 29736/ug = Hs.75627/len = 1356
Genes marked with asterisk (*) met the criteria for inclusion in the master list of stromal-cell genes (Table 23).		

TABLE 22B

List of genes from Table 3 with Affy data & group statistics									
Gene name	CD45	CD34	CD19	CD20	CD22	*CD10 (CALLA)	*TCF3 (E2A)	*CD79A (IGBP1)	*HLA class II, Dr alpha
Probe ID	40518_at	538_at	1116_at	619_s_at	38521_at	1389_at	1373_at	34391_at	37039_at
UNFRA	1628	1804	1157.7	74.6	1396.2	7030.2	1619.4	1226	38062.1
Signal									
UNFRA	P	A	A	A	A	P	P	P	P
Detection									
UNFRB	6183.6	2590	148.6	154.7	305.6	6009	2268.3	1983.7	132286.8
Signal									
UNFRB	P	A	A	A	A	P	P	P	P
Detection									
UNFRBR1	7040.1	1745.9	967.7	52.7	1213.7	7322.8	1347.9	2033.4	84235.4
Signal									
UNFRBR1	P	P	A	A	A	P	P	P	P
Detection									
UNFRBR2	8639.9	1465.7	429.1	125.3	1362.8	5749.1	1111	2176	78133.5



TABLE 22B-continued

List of genes from Table 3 with Affy data & group statistics									
SCD3 Detection	P	A	P	P	A	P	P	P	P
			Gene name	*HLA class II, Dr beta 1	*B2 microglobulin	*BMI1	CD2	CD3 epsilon	
			Probe ID	33261_at	34644_at	41562_at	40738_at	36277_at	
			UNFRA	3806.9	38013.9	5103.1	459.5	1951.2	
			Signal						
			UNFRA	P	P	P	A	M	
			Detection						
			UNFRB	14982.3	30293	5336.3	1169.5	1115.6	
			Signal						
			UNFRB	P	P	P	A	A	
			Detection						
			UNFRBR1	25062.7	55572.2	4871.4	575	967.4	
			Signal						
			UNFRBR1	P	P	P	A	A	
			Detection						
			UNFRBR2	24972.2	48428.8	5217.7	387.1	885.5	
			Signal						
			UNFRBR2	P	P	P	P	P	
			Detection						
		Collective	UNFRCR1	4675.3	52157	3429.9	988.7	985.3	
		USC	Signal						
			UNFRCR1	P	P	P	M	A	
			Detection						
			UNFRCR2	4462.9	56560	4013.6	512.9	996.4	
			Signal						
			UNFRCR2	P	P	P	P	P	
			Detection						
			UNFRDR1	11076.1	63105.6	4455.3	802.8	1802.9	
			Signal						
			UNFRDR1	P	P	P	A	M	
			Detection						
			UNFRDR2	6802.8	48011.9	5004.1	892.4	1753.6	
			Signal						
			UNFRDR2	P	P	P	A	P	
			Detection						
			MPCA	6685.1	64828.1	6263.5	754.2	918.2	
			Signal						
			MPCA	P	P	P	P	A	
			Detection						
			MPCBR2	2627.9	39862.2	4765.5	349	1220.4	
			Signal						
			MPCBR2	P	P	P	A	P	
			Detection						
		Collective	MPCCR2	2497.2	59113.3	4148.6	485.8	1005.6	
		MPC	Signal						
			MPCCR2	P	P	P	A	A	
			Detection						
			MPCDR1	7326.5	57360.7	3307.1	960.6	1709	
			Signal						
			MPCDR1	P	P	P	A	P	
			Detection						
			MPCDR2	8206	41709	3629	793.8	2143.1	
			Signal						
			MPCDR2	P	P	P	A	P	
			Detection						
			SCA1	1845.1	29462.7	28699.3	667.6	1305.2	
			Signal						
			SCA1	P	P	P	A	P	
			Detection						
			SCA2	2295.9	21558.1	27214.8	953.1	1203.5	
			Signal						
			SCA2	P	P	P	A	M	
			Detection						
			SCA3	1884.2	43650.9	15236.4	491.8	495.4	
			Signal						
			SCA3	P	P	P	A	A	
			Detection						
			SCB1	2385.2	25705.2	22346	1454.1	763	
			Signal						
			SCB1	P	P	P	A	A	
			Detection						
			SCB3	2897.4	22892.5	30792.8	776.2	1162.4	
			Signal						



TABLE 22B-continued

List of genes from Table 3 with Affy data & group statistics						
Single cell MPC	SCB3 Detection	P	P	P	A	P
	SCC1 Signal	8143.9	33666.2	28528.7	1345.6	2730.5
	SCC1 Detection	P	P	P	P	A
	SCC3 Signal	6245.5	40680.9	30696.4	741.5	2316.8
	SCC3 Detection	P	P	P	A	M
	SCD1 Signal	5042.5	43517.2	29022.8	752.8	1730.5
	SCD1 Detection	P	P	P	M	A
	SCD2 Signal	3599.8	43695	24371.8	807.1	1087.7
	SCD2 Detection	P	P	P	M	M
	SCD3 Signal	3524.7	43333.9	29904.1	736.3	1004.2
	SCD3 Detection	P	P	P	P	P

Genes marked with asterisk (\*) met the criteria for inclusion in the master list of stromal-cell genes (Table 23).

TABLE 22C

Gene name	CD5	CD7	CD13	CD33	CD14
Probe ID	32953_at	771_s_at	39385_at	36802_at	36661_s_a
UNFRA	143.2	648.9	16071.7	497.7	6674.2
UNFRA Signal	A	A	P	A	P
UNFRA Detection	159.4	836.4	10077.1	1953.1	37273.3
UNFRB	A	A	P	M	P
UNFRB Signal	70.3	859.9	7365.8	1294.9	22119.4
UNFRB Detection	A	A	P	P	P
UNFRBR1	62.6	432.1	6525.3	1016.3	19788.3
UNFRBR1 Signal	A	A	P	P	P
UNFRBR1 Detection	A	A	P	P	P
UNFRBR2	62.6	432.1	6525.3	1016.3	19788.3
UNFRBR2 Signal	A	A	P	P	P
UNFRBR2 Detection	A	A	P	P	P
Collective USC					
UNFRCR1	137	938.3	14153	474.5	10012.2
UNFRCR1 Signal	A	A	P	A	P
UNFRCR1 Detection	157.5	364.9	9686.8	431.7	6424.2
UNFRCR2	A	A	P	M	P
UNFRCR2 Signal	264.7	731.5	7656.6	1417	14154
UNFRCR2 Detection	A	A	P	P	P
UNFRDR1	245.3	1383.6	9577.5	708.2	14135.1
UNFRDR1 Signal	A	A	P	P	P
UNFRDR1 Detection	A	A	P	P	P
UNFRDR2	83.7	503.6	7855.1	328.2	4253.8
UNFRDR2 Signal	A	A	P	A	P
UNFRDR2 Detection	A	A	P	A	P
MPCA	103.2	365.9	2099.1	428	262.3
MPCA Signal	A	A	P	A	A
MPCA Detection	A	A	P	A	A
MPCBR2	103.2	365.9	2099.1	428	262.3
MPCBR2 Signal	A	A	P	A	A
MPCBR2 Detection	A	A	P	A	A

TABLE 22C-continued

Gene name	CD5	CD7	CD13	CD33	CD14
<u>Collective MPC</u>					
MPCCR2	70.1	464	16492.9	320.6	1291.2
Signal					
MPCCR2	A	A	P	A	A
Detection					
MPCDR1	174.5	823.9	15377.2	669.2	10550
Signal					
MPCDR1	A	A	P	P	P
Detection					
MPCDR2	213.2	600.7	10487.7	890.8	11953
Signal					
MPCDR2	A	A	P	P	P
Detection					
SCA1	96.1	61.1	1611.5	70.8	133.5
Signal					
SCA1	A	A	A	A	A
Detection					
SCA2	77.9	606	2433.8	376.6	165.3
Signal					
SCA2	A	A	A	A	A
Detection					
SCA3	78.8	145.1	2391.8	643.5	100.9
Signal					
SCA3	A	A	A	A	A
Detection					
SCB1	157.5	427.6	1100.4	134.2	156
Signal					
SCB1	A	A	A	A	A
Detection					
SCB3	145.3	297.5	1164.7	116.3	179.7
Signal					
<u>Single cell MPC</u>					
SCB3	A	A	A	A	A
Detection					
SCC1	435.9	281.7	306.4	406	369.1
Signal					
SCC1	A	A	A	A	A
Detection					
SCC3	166.7	622.2	358.3	107.5	146.3
Signal					
SCC3	A	A	A	A	A
Detection					
SCD1	231.3	539.1	1250.4	580.7	115.8
Signal					
SCD1	A	A	A	A	A
Detection					
SCD2	241.4	189.4	1371.8	432.1	194.9
Signal					
SCD2	A	A	A	A	A
Detection					
SCD3	75.6	295.9	1597.5	104.3	501.4
Signal					
SCD3	A	A	A	A	A
Detection					

TABLE 22D

Gene Name	Collective USC	Collective MPC Mean	Single cell MPC	Collective USC	Collective MPC Standard Deviation	Single cell MPC
CD45	4,369.25	1,199.86	5,500.54	2,680.72	748.751	5,988.78
CD34	1,925.09	1,650.18	1,019.14	515.294	679.755	460.488
CD19	778.075	963.06	10,449.40	388.856	377.621	8,901.83
CD20	334.825	402.02	782.01	363.374	441.342	445.444
CD22	1,620.86	1,456.00	1,117.22	682.519	264.292	531.154
*CD10 (CALLA)	10,063.43	9,360.68	25,547.93	4,050.99	1,759.27	11,613.00
*TCF3 (E2A)	1,617.35	1,647.30	2,590.47	369.25	542.922	1,390.68
*CD79A (IGBP1)	1,886.08	2,099.60	1,725.52	371.138	302.356	632.964

TABLE 22D-continued

Gene Name	Collective USC	Collective MPC Mean	Single cell MPC	Collective USC	Collective MPC Standard Deviation	Single cell MPC
*HLA class II, Dr alpha	62,391.46	21,008.50	6,140.54	34,143.40	14,681.30	4,823.50
*HLA class II, Dr beta 1	11,980.15	5,468.54	3,786.42	8,890.23	2,707.57	2,083.76
*B2 microglobulin	48,892.80	52,574.66	34,816.26	10,578.15	11,129.65	9,252.53
*BMI1	4,678.93	4,422.74	26,681.31	665.245	1,167.87	4,850.87
CD2	723.488	668.68	872.57	281.153	246.894	301.695
CD3 epsilon	1,307.24	1,399.26	1,379.92	445.605	516.627	691.075
CD5	155	128.94	170.65	72.1	61.941	111.307
CD7	774.45	551.62	346.56	318.835	173.94	195.186
CD13	10,139.23	10,462.40	1,358.66	3,353.99	5,858.02	712.465
CD33	974.175	527.36	297.2	547.821	247.232	216.123
CD14	16,322.59	5,662.06	206.29	10,176.48	5,331.81	127.73

Attached hereto is a compact disk and duplicate copy (labeled "Copy 1" and "Copy 2," respectively) containing the file "Table 23.txt", created on Jul. 9, 2003, the size of which is 1,124 kilobytes, and the file "Table 24.txt", created on Jul. 9, 2003, the size of which is 4,716 kilobytes, the contents of each of which is incorporated herein by reference in its entirety. Tables 23 and 24 are contained within the indicated files in accordance with 37 C.F.R. §§ 1.52 and 1.58.

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 I claim:  
 1. A method for enhancing engraftment of hematopoietic cells in a mammal, said method comprising administering to the mammal:  
 (a) mononuclear cells comprising hematopoietic cells, and  
 (b) isolated pluri-differentiated mesenchymal progenitor cells, wherein the isolated pluri-differentiated mesenchymal progenitor cells are obtained directly from a primary Dexter cell culture, wherein each of the pluri-differentiated mesenchymal progenitor cells simultaneously expresses a plurality of genes that are markers for multiple cell lineages, wherein the multiple cell lineages comprise at least four different mesenchymal cell lineages, wherein each of the markers is specific for a single cell lineage, and wherein the isolated pluri-differentiated mesenchymal progenitor cells are administered in an amount effective to enhance engraftment of the hematopoietic cells.  
 2. The method of claim 1, wherein the isolated pluri-differentiated mesenchymal progenitor cells are administered by intravenous injection or by injecting directly to the site of intended activity.

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3. The method of claim 1, wherein the mononuclear cells are administered concurrently with the isolated pluri-differentiated mesenchymal progenitor cells.

4. The method of claim 1, wherein the mononuclear cells comprise hematopoietic progenitor cells.

5. The method of claim 1, wherein the isolated pluri-differentiated mesenchymal progenitor cells and the mononuclear cells are administered to the mammal in a cell suspension.

6. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are not cells of a cell line.

7. The method of claim 1, wherein that at least four different mesenchymal cell lineages comprise adipocyte, osteoblast, fibroblast, and muscle cell.

8. The method of claim 1, wherein the markers specific for a single cell lineage are selected from the group consisting of Nile Red, Oil Red O, adipsin, alkaline phosphatase, cadherin-11, chondroitin sulfate, collagen type I, decorin, fibronectin, prolyl-4-hydroxylase, actin, caldesmon, and transgelin.

9. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are not neoplastic cells.

10. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are chromosomally normal, as determined by Geimsa-trysin-Wrights (GTW) banding.

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11. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are human cells.

12. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are obtained by providing a cell culture preparation by the Dexter method, treating the cells of the cell culture preparation to obtain a cell suspension, removing macrophages from the cell suspension, fractionating the remaining cells, and collecting the fraction of cells containing said isolated cell.

13. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are not immortalized.

14. The method of claim 1, wherein the mammal is human.

15. The method of claim 1, wherein the pluri-differentiated mesenchymal progenitor cells are obtained from the mammal prior to said administering.

16. The method of claim 1, wherein the mononuclear cells are human cells.

17. The method of claim 1, wherein the mononuclear cells are bone marrow mononuclear cells.

18. The method of claim 1, wherein the mononuclear cells are human cells, and wherein the mammal is human.

19. The method of claim 1, wherein the mononuclear cells and the pluri-differentiated mesenchymal progenitor cells are human cells, and wherein the mammal is human.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,442,390 B2  
APPLICATION NO. : 10/887582  
DATED : October 28, 2008  
INVENTOR(S) : Beerelli Seshi

Page 1 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page,

Item (57) Abstract, line 8, "invention also method" should read  
--invention also discloses a method--.

Column 1,

Line 37, "Accordingly. the government" should read --Accordingly, the government--.

Column 5,

Line 49, "form alin-fixed," should read --formalin-fixed,--.

Column 6,

Lines 64-65, "hematopoletic cells" should read --hematopoietic cells--.

Column 8,

Line 18, "(ID (538\_at)," should read --(ID 538\_at)--.

Line 23, "IILA-Dr (ID 33261\_at)" should read --HLA-Dr (ID 33261\_at)--.

Column 26,

Line 33, "includes a "physiological" should read --includes a physiological--.

Column 36,

Line 39, "DNA (p177H8)" should read --DNA (p17H8)--.

Line 55, "Lanes 12, 13. were" should read --Lanes 12, 13 were--.

Column 37,

Lines 8-11, "lanes 12 and 13.

Increased Survival and Evidence of MPC Effect on GvHD

Increased Survival and Evidence of MPC Effect on GvHD:

FIGS. 13A-1, 13A-2," should read

--lanes 12 and 13.

Increased Survival and Evidence of MPC Effect on GvHD:

FIGURES 13A-1, 13A-2,--.

Column 41,

Line 60, "INIB *Homo sapiens*" should read --INIB *Homo sapiens*--.



UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,442,390 B2  
APPLICATION NO. : 10/887582  
DATED : October 28, 2008  
INVENTOR(S) : Beerelli Seshi

Page 2 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 42,

Lines 18-19, "menus Filter→AutoFilter→Customs enter key words (e.g., cell adhesion or cell cycle) OK" should read  
--menu→Filter→AutoFilter→Custom→enter key words (e.g., cell adhesion or cell cycle) →OK--.

Line 39, "Results" should read --Results:--.

Column 52,

Lines 30-31, "1<sup>st</sup> (Amersham Pharmacia Biotech)." should read  
--1<sup>st</sup> D (Amersham Pharmacia Biotech).--.

Column 58,

Line 48, "ALL, MM) A" should read --ALL, MM): A--.

Column 61,

Lines 48-49, "samples AND 7 of 8 collective USC samples AND 9 of 10" should read  
--samples and 7 of 8 collective USC samples and 9 of 10--.

Column 62,

Line 17, "CD20±, (CD10+," should read --CD20±, CD10+,--.  
Line 62, "nonhematopoietic" should read --nonhematopoietic--.

Column 64,

Line 7, "Conclusions The findings" should read --Conclusions: The findings--.

Column 72,

Line 33, "FIGS. 21A-21F shows" should read --Figures 21A-21F show--.  
Line 40, "(ID (538\_at)," should read --(ID 538\_at),--.

Column 85,

Table 7, row for "41449\_at"

"41449_at	SGCE	AJ000534	sarcoglycan, epsilon	Dystonia, myclonic
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should read

--41449_at	SGCE	AJ000534	sarcoglycan, epsilon	Dystonia, myclonic
40953_at	CNN3	S80562	calponin 3, acidic	

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INVENTOR(S) : Beerelli Seshi

Page 3 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 91,

Row for “159\_at” and footnote to Table 11,

“159\_at VEGFC U43142      vascular endothelial growth factor C / ligand and  
activator of  
the receptor  
tyrosine  
kinase Flt4  
”

should read

--159\_at VEGFC U43142      vascular endothelial growth factor C / ligand and  
activator of  
the receptor  
tyrosine  
kinase Flt4

Footnote: Stromal cell gene list contains a number of genes that are potentially capable of causing endothelial differentiation and vasculogenesis within the marrow microenvironment; however, these genes themselves are not necessarily endothelial cell markers. In fact, stromal cells express a gene, EDF1, the expression of which inversely correlates with endothelial cell differentiation within the stromal cells, suggesting that the endothelial cell pathway is being actively “turned off” in these cells.--.

Column 97,

Table 13, row “38388\_at” for “Description” column, “A zinc fanger protein” should read  
--A zinc finger protein--.

Column 100,

Table 13, row “35226\_at” for “Phenotype/Function” column, “participates inconnective tissue patterning.” should read  
--participates in connective tissue patterning.--.

Column 103,

Table 14, row “35150\_at” for “Description” column, “all mature B cels,” should read  
--all mature B cells--.

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 7,442,390 B2  
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DATED : October 28, 2008  
INVENTOR(S) : Beerelli Seshi

Page 4 of 4

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 109,

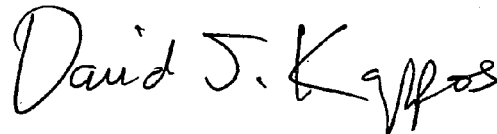
Table 19, "Cell lineage" column, line 26, "Hematopoietic" should read  
--Hematopoietic--.

Column 128,

Line 47, "A method for enhancing engraftment" should read  
--A method for enhancing engraftment--.

Signed and Sealed this

Twenty-ninth Day of September, 2009

A handwritten signature in black ink that reads "David J. Kappos". The signature is written in a cursive, flowing style with a large initial 'D' and a stylized 'K'.

David J. Kappos  
*Director of the United States Patent and Trademark Office*