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Isolation of Mesenchymal Stem Cells Derived from Adult Bone Marrow and Umbilical Cord Blood and Their Potential to Differentiate into Osteoblasts

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ISOLATION OF MESENCHYMAL STEM CELLS DERIVED FROM ADULT BONE MARROW AND UMBILICAL CORD BLOOD AND THEIR POTENTIAL TO DIFFERENTIATE INTO OSTEOBLASTS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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LIST OF ABBREVIATIONS

HSC	hematopoietic stem cell
NSC	neural stem cell
ES cell	embryonic stem cell
MSC	mesenchymal stem cell
MNC	mononuclear cell
ABM	adult bone marrow
UCB	umbilical cord blood
GVHD	graft-versus-host disease
HLA	human lymphocyte antigen
CD	cluster of differentiation
DNA	deoxyribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
Sca-1	stem cell antigen-1
LNGFR	low affinity nerve growth factor receptor
MACS	magnetic-activated cell sorting
APC	antigen-presenting cell
DMEM	Dulbecco's modified eagle medium
FBS	fetal bovine serum
β-FGF	basic-fibroblast growth factor
EDTA	ethylenediaminetetraacetic acid
PBS	phosphate-buffered solution
PE	phycoerythrin
FITC	fluorescein isothiocyanate
FACS	flow activated cells sorting
CBFA-1	core-binding factor α-1
Dex	dexamethasone
βGP	β-glycerol phosphate
mRNA	messenger ribonucleic acid
Osx	osterix
AA	ascorbic acid
TBS	tris-buffered saline
DEPC	diethyl pyrocarbonate
Ab	antibody
MW	molecular weight
BMP	bone morphogenic protein
CNS	central nervous system

ABSTRACT

ISOLATION OF MESENCHYMAL STEM CELLS DERIVED FROM ADULT BONE MARROW AND UMBILICAL CORD BLOOD AND THEIR POTENTIAL TO DIFFERENTIATE INTO OSTEOBLASTS

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The demand for treatment strategies of musculoskeletal tissues is continuously growing, especially considering the increasing number of elderly people with degenerative diseases of the skeletal system. Despite major strides in the field of bone regenerative medicine during the years, current therapies, such as bone grafts, still have several limitations. Multipotent stem cells, such as mesenchymal stem cells (MSCs) are promising candidates for tissue repair because of their differentiation potential and their capacity to undergo extensive replication. However, isolating a homogeneous population of MSCs from multiple sources is an area that needs to be addressed. Also,

the knowledge regarding the mechanisms and pathways that lead to the final osteogenic differentiation is still scarce.

The following research is a feasibility study on a new isolation technique developed by our lab. The major focus of the research will be the isolation and characterization of mesenchymal stem cells from both adult bone marrow and umbilical cord blood using a novel isolation method based on immunodepletion. Furthermore we will look at the potential of these isolated MSCs to differentiate into mature, bone producing osteoblasts.

The results of the studies showed that our novel isolation method allowed proliferation of a homogeneous MSC population. Our immunodepleted MSCs were 99% double positive for antibodies CD44 and CD105 which are highly specific for multipotent MSCs while cells isolated using the plastic adherence method were only 43% double positive for the two MSC-specific markers. Homogeneous MSCs were derived from both adult bone marrow and umbilical cord blood using our isolation method. Utilizing the techniques of confocal microscopy, von Kossa staining, and RT-PCR we also show that MSCs, upon stimulation with osteogenic supplements, differentiate into osteoblasts capable of being used for bone tissue engineering applications.

CHAPTER 1

GENERAL INTRODUCTION

A stem cell is a special kind of cell that has a unique capacity to renew itself and to give rise to specialized cell types. Although most cells of the body, such as heart cells or skin cells, are committed to conduct a specific function, a stem cell is uncommitted and remains uncommitted, until it receives a host of signals to develop into a specialized cell. Their proliferative capacity combined with their ability to become specialized makes stem cells unique. Stem cells that can develop into any cell type in the body are referred to as pluripotent, because the cells have the potential to develop almost all of the more than 200 different known cell types [1]. Stem cells have the ability to act as a repair system for the body, because they can divide and differentiate, replenishing other cells as long as the host organism is alive.

Stem Cell Populations in Embryos and Adults

Embryonic Stem Cells

Embryonic stem (ES) cells are cells that are undifferentiated but have the capacity to differentiate into all adult tissues. ES cells can be cultured for extended periods and genetically manipulated without loss of their pluripotential capacity, as assessed by their ability to participate in all aspects of the development of the embryo proper when reintroduced in vivo [2]. ES cells can be induced to differentiate in vitro into two-or three-dimensional (embryoid bodies) cultures containing cells from all three

embryonic lineages (ectoderm, mesoderm, and endoderm), including cells of the hematopoietic [3], endothelial [4], cardiac [5, 6], and neuronal [7, 8] tissues [9].

Tissue-Specific Stem Cells

Hematopoietic Stem Cells (HSCs)

A hematopoietic stem cell is a cell isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can migrate out of the bone marrow into circulating blood, and can undergo programmed cell death, called apoptosis—a process by which cells that are detrimental or unneeded self-destruct. HSCs are responsible for forming the immune cells and the constant renewal of the blood by producing billions of new blood cells each day. Experimental evidence supporting the existence of such cells was first provided by Till &McCulloch [10], who injected lethally irradiated mice with bone marrow-derived cells from healthy syngenic (genetically identical) donors and observed the formation in the spleen of multilineage colonies that could be shown to be derived from single injected cells. Subsequent experiments utilizing genetic marking have demonstrated that long-term engraftment of both the lymphoid and myeloid lineages can be achieved by the progeny of a single cell [11, 12]. In addition, the ability to repopulate multiple secondary recipients with the progeny of a single clone, along with their extensive amplification when limiting numbers are transplanted, has demonstrated that these very primitive cells are capable of extensive (>1000-fold) in vivo amplification [13, 14].

Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells, also known as marrow stromal cells or mesenchymal progenitor cells, are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct mesenchymal lineages [15]. To date, MSCs of multiple adult vertebrate species have been demonstrated to differentiate into lineage-specific cells that form bone, cartilage, fat, tendon, and muscle tissue [16-18]. In addition to differentiation into their natural derivatives, MSCs have the potential to differentiate into other types of tissue-forming cells such as hepatic, renal, cardiac, and neural cells. The descriptive terms "pluripotent" or "multipotent" are reciprocally used to describe the capacity of MSCs to differentiate into a wide arrange of mammalian tissues [19].

Sources of Mesenchymal Stem Cells

Bone Marrow

Bone marrow is a complex tissue comprised of hematopoietic precursors, their differentiated progeny, and a connective tissue network referred to as stroma. The stroma itself is a heterogeneous mixture of cells including adipocytes, reticulocytes, endothelial cells, and fibroblastic cells which are in direct contact with the hematopoietic elements [20]. Since it has been well established that the stroma contains cells that differentiate into bone, cartilage, fat, and a connective tissue which supports the differentiation of hematopoietic stem cells [21-23], identification of the progenitor cells for these mesenchymal tissues has been an area of active investigation.

Friedenstein [24] and others [25-27] have demonstrated that culture-adherent cells present in the marrow stroma are capable of differentiating into bone and cartilage when placed into an appropriate environment in vivo. These experiments have led to the hypothesis that stroma contains a unique population of stem cells which are capable of differentiating along multiple mesenchymal cell lineages [15, 26, 27].

Currently, bone marrow (BM) represents the main source of MSCs for both experimental and clinical studies [18, 28, 29], but aspirating bone marrow from the patient is an invasive procedure and, in addition, it has been demonstrated that the number and the differentiating potential of bone marrow MSCs decreases with age [30]. Therefore, it can be argued that cells with both extensive potency of proliferation and differentiation would represent an optimal tool for future cell-based therapeutic applications.

Umbilical Cord Blood

Umbilical cord blood (UCB) has turned out to be an excellent alternative source of hematopoietic stem cells (HSCs) for clinical-scale allogenic transplantation [31]. Cord blood represents a relatively rich source of otherwise discarded HSCs and 80ml can reconstitute the hematopoietic system in small patients, usually children, although there are some reports of successful transplantation of adults [32, 33]. Cord blood has a reduced risk of graft versus host disease (GVHD) compared with bone marrow in both HLA-matched and mismatched HSC transplants, due to naiveté of T lymphocytes in cord blood [34]. Since the first successful related cord blood transplant 16 years ago,

the efficacy of cord HSCs has been demonstrated in over 2000 unrelated and related cord blood transplants worldwide, even in cases of incomplete tissue type matching between donor and recipient. For adults with malignant and non-malignant disease, cord blood is a good alternative source of HSCs to bone marrow or cytokine (granulocyte colony-stimulating factor) stimulated peripheral blood stem cells. Unrelated cord blood transplants result in good outcome in children transplanted in remission and with inborn errors of metabolism and immune deficiencies, which in part relates to the small size of these patients.

Essential preclinical studies proved a higher percentage of $CD34^{+}$ $CD38^{-}$ cells in UCB compared with BM, suggesting that more primitive progenitors may be abundant in neonatal blood [35]. The same might apply for the presence of MSCs or progenitor cells. Lee et al. [36] reported it is possible to obtain clonally expanded MSCs from umbilical cord blood with remarkable potential to differentiate into multiple lineages of mesodermal and nonmesodermal origin.

Telomere Length is Linked to Limited Proliferation Capacity

Telomeres, the terminal guanine-rich sequences of chromosomes, are structures that function in the stabilization of the chromosome during replication by protecting the chromosome end against exonucleases. Telomeres are reduced in length during each cell division. The gradual decrease in telomere length may function as a timing mechanism that, when reaching a critical length, signals a cell to stop dividing and to enter cellular senescence. A clear correlation is seen between replicative capacity and the initial telomere length in normal somatic cells [37]. Human telomeres, which consist of repeats of the sequence TTAGGG at the chromosome ends, are elongated by the ribonucleoprotein enzyme telomerase. Telomerase is preferentially expressed in germline cells, immortalized cells, and in most tumor cells. Thus, in these cells, telomerase apparently balances the telomere loss with de novo synthesis of telomeric DNA. In contrast, telomerase is not expressed in most human somatic cells, and the cells become senescent when progressive telomere shortening during each cell division reaches a threshold telomere length. Cells in regenerative tissues such as skin and bone also could decrease their telomere length and replicative capacity with aging, because their progenitors also divide and decrease their telomere length throughout the life span of cells. Thus, during aging, the cumulative numbers of senescent progenitor or mature cells in renewable tissue may contribute to disorders in tissue repair or remodeling, resulting in organismic senescence [37].

With advancing age, a progressive and age-dependent bone loss can be observed in

patients with systemic or localized bone diseases. This bone loss may be caused by, at least in part, a reduced bone mass that most likely results from inadequate bone formation by osteoblasts. The rate of bone formation is largely determined by the number of osteoblasts, which in turn is determined by the rate of replication of progenitors (MSCs) and the life span of mature cells. Changes in either the replicative potential or the life span of osteoblasts may alter the rate of bone formation.

Applications of Mesenchymal Stem Cells in Tissue Engineering and Regenerative Medicine

Bone is a dynamic, highly vascularized tissue with a unique capacity to heal and remodel without leaving a scar. These properties, together with its capacity to rapidly mobilize mineral stores on metabolic demand, make it the ultimate smart material. Its main role is to provide structural support for the body. Furthermore the skeleton also serves as a mineral reservoir, supports muscular contraction resulting in motion, withstands load bearing and protects internal organs [38]. Hence, it is logical to say that major alterations in its structure due to injury or disease can dramatically alter one's body equilibrium and quality of life.

The demand for treatment strategies of musculoskeletal tissues is continuously growing, especially considering the increasing number of elder people with degenerative diseases of the skeletal system. In the USA alone, the population over the age of 65 is expected to double and the number of people over 85 is projected to quadruple in the next 20 years [39]. Every year 110,000 Americans suffer nonhealing

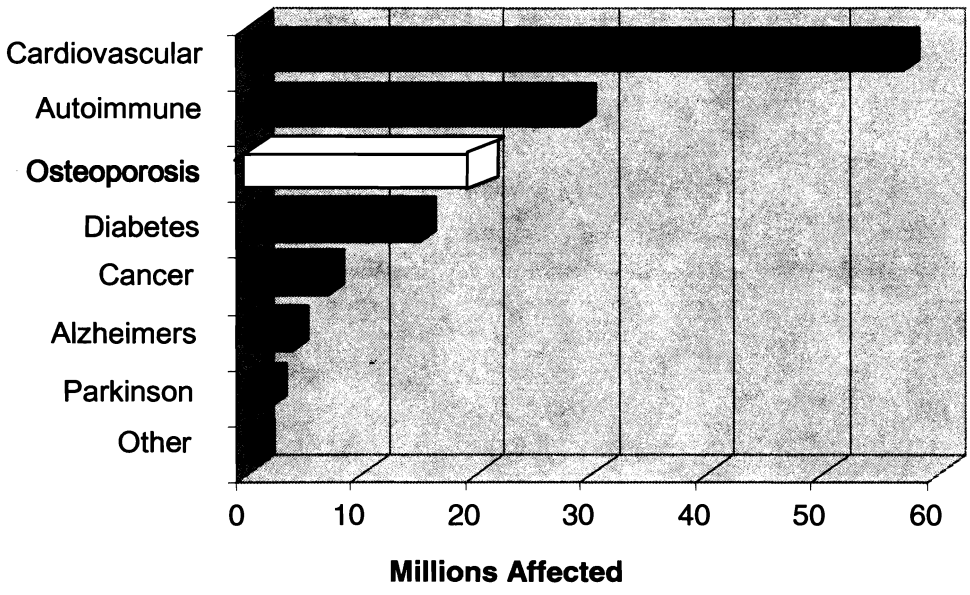
long bone defects. For classical skeletal reconstruction, orthopaedic surgeons have applied autologous (derived from the patient's own cells) or allogenic (same species, but not patient's own cells) tissues and artificial alloplastic implants. The success of such surgeries is limited due to the donor site morbidity of autologous grafts, the immunogenicity of allogenic grafts and loosening of the alloplastic implants [40].

Although major progresses were done in the field of bone regenerative medicine during the years, current therapies, such as bone grafts, still have several limitations. Moreover, and despite of the fact that materials science technology has resulted in clear improvements in the field of bone substitution medicine, no adequate bone substitute has been developed. Thus, most of the severe injuries related to bone are still unrecoverable or not adequately treated.

Promising new bioengineering technologies, such as tissue engineering, which is an interdisciplinary field of physicians, engineers and scientists, may provide novel tools for reconstructive surgery and will probably help to overcome the previously mentioned disadvantages. Tissue engineering allows the design of functionally active cells within supportive bio-scaffolds combined with the controlled delivery of growth factors to promote the development of new tissues such as cartilage and bone for the restoration of pathologically altered tissues [41]. Consequently, common tissue engineering principles focus on: (1) healthy cells, which have to be nonimmunogenic, easy to isolate and highly responsive to distinct environmental cues, (2) suitable carriers for the in vitro cell differentiation and subsequent transplantation, and (3) a set of defined bioactive molecules driving the process of differentiation and maturation. Bone

tissue engineering requires the appropriate osteoprecursor cells and a host of osteoinductive factors.

The proliferation capacity of many adult organ-specific cells is low and long-term in vitro cultivation, in particular, reduces their functional quality. These are the reasons why so much attention has been drawn to pluripotent embryonic (ES) or multipotent stem cells. Multipotent stem cells, such as mesenchymal stem cells (MSCs) are promising candidates for tissue repair because of their differentiation potential and their capacity to undergo extensive replication. Sources of these cells include the embryo, umbilical cord blood, amniotic fluid and certain sites in adults such as the central nervous system (CNS) and bone marrow. More than 100 million Americans suffering from diseases might profit from stem cell-based tissue engineering applications. Mesenchymal stem cells induced into osteoblasts hold promise for healing musculoskeletal disorders such as non-union fractures and osteoporosis (**Figure 1**).


Cardiovascular
Autoimmune
Osteoporosis
Diabetes
Cancer
Alzheimers
Parkinson
Other
0
10
20
30
40
50
60
Millions Affected

One of the early problems regarding the study of MSCs was the high heterogeneity of whole bone marrow cultures. In 1992 Haynesworth et al. [42] described a method that, although did not completely solve the heterogeneity problems of the cultures, was able to overcome some of the problems of previous techniques. The methodology is based on the separation of the MSCs through gradient centrifugation, after which cells were plated on tissue culture plastic. In these conditions they presented similar fibroblastic morphology and the same characteristics of the cells described by Friedenstein [24]. In later studies published by several authors these cells were able to develop into distinct terminal and differentiated cells including bone, cartilage, fat, and tendon.

Besides its differentiation potential, MSCs present other important properties. As described by Bruder et al. [20] they can be extensively expanded in vitro. Pittinger et al. [18] also showed that with an increased number of passages they did not spontaneously differentiate. Furthermore it has been suggested that these cells may possess immunosuppressive effects which may render them either "immune privileged" or perhaps immunosuppressive in vivo, which would make them suitable for allogenic or xenogenic transplantation.

Although MSCs have several advantages regarding their use for tissue engineering, there are still some issues that need to be addressed. For instance, it is known that the percentage of MSCs present in the bone marrow is very low (1 in every 100,000 cells) [43] which would make the expansion time consuming. New expansion methods can be the solution to obtain the amount of cells necessary to be useful in

clinical applications. The differentiation capability of donors from different ages also needs to be addressed. It has been shown that the numbers as well as the differentiation potential of MSCs was somewhat diminished when these were isolated from elderly patients [30, 44]. Finally, like in the ES cells, the knowledge regarding the mechanisms and pathways that lead to the final osteogenic differentiation is still scarce.

Overall it can be said that, for now, MSCs represent more advantages than ES cells for use in bone tissue engineering. The former are already in clinical trials for certain applications, including bone tissue engineering while the latter still have a long way until they reach that stage.

Focus of current research

The major focus of the following research will be the isolation and characterization of mesenchymal stem cells derived from both adult bone marrow and umbilical cord blood. Using a novel isolation method based on positive and negative selection we look to isolate a more homogeneous MSC population when compared to the widely used plastic adherence method. We hypothesize that with the use of specific cell-surface markers we can remove unwanted cells from the samples and extensively grow, *in vitro*, a purer population of mesenchymal stem cells. Furthermore we will look at the potential of these isolated MSCs to differentiate into mature, bone producing osteoblasts. Using the techniques of confocal microscopy, cellular staining, and RT-PCR we will show that MSCs, upon stimulation, can differentiate into osteoblasts capable of being used for bone tissue engineering applications.

CHAPTER 2

This chapter will focus on the isolation and characterization of a homogeneous mesenchymal stem cell (MSC) population from both adult bone marrow and umbilical cord blood using a novel isolation method. We hypothesize that an immuno-depletion method against specific cell-surface markers will increase MSC homogeneity compared to the widely used plastic adherence method.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STEM CELLS FROM HUMAN BONE MARROW AND UMBILICAL CORD BLOOD

ABSTRACT

This study compares the isolation and characterization of mesenchymal stem cells from two different sources, bone marrow and umbilical cord blood. In order to study the molecular mechanisms and therapeutic potential of MSCs, a homogeneous cell population must be isolated and characterized. A novel isolation technique developed by our lab is compared to the frequently used method of plastic adherence.

With the use of immuno-depletion we removed unwanted cells from our samples by attaching paramagnetic antibodies targeted to cells to be removed. Our results show that after 4 weeks of proliferation in MSC expansion media, the immuno-depleted MSCs were 99% double positive for antibodies CD44 and CD105 which are highly specific for multipotent MSCs. Cells isolated using the plastic adherence method were only 43% double positive for the two MSC-specific markers.

With a homogeneous population of MSCs we looked at the similarities and differences between MSCs isolated from adult bone marrow and umbilical cord blood. The morphology of the MSCs from each source varied slightly as the adult bone marrow (ABM)-derived MSCs were all thin with long spindles while umbilical cord blood (UCB)-derived MSCs ranged from flat, round cells to thin, elongated MSCs.

However, upon confluency, both ABM-derived and UCB-derived MSCs displayed homogeneous fibroblastoid morphologies with long protrusions at both poles.

INTRODUCTION

Mesenchymal stem cells (MSCs) can be enriched from whole bone marrow and umbilical cord blood by isolation of fibroblastoid cells via their preferential attachment to tissue culture plastic. Although this fractionation method yields a phenotypically and functionally heterogeneous fibroblastoid cell population [45, 46] it has been used widespread due to the lack of specific antigens for direct isolation of MSCs from both sources. MSC colonies isolated by adherence to the plastic culture plate are heterogeneous, likely containing osteoblasts and/or osteoprogenitor cells, fat cells, fibroblasts, reticular cells, macrophages, endothelial cells, and a fraction of blood cells and hematopoietic stem cells [47]. Hematopoietic cells constitute a large percentage of plastic adherent murine marrow cultures due to their ability to adhere directly to tissue culture plastic as well as bind to fibroblastoid (stromal) cells via engagement with adhesion molecules, cytokine receptors, and extra cellular matrix proteins [48-51]. Moreover, hematopoietic cells persist in these cultures even after serial passage due to the ability of stromal cells to support granulopoiesis and B cell lymphopoiesis even in the absence of exogenous growth factors and cytokines [46, 52]. Several methods have been reported to separate fibroblastoid and hematopoietic cells in plastic adherent cell cultures. Wang and Wolf [53] demonstrated that when bone marrow cells are plated at very low density, fibroblastoid colonies arise that are devoid of hematopoietic cell types. However, the method is impractical as a purification scheme because it yielded only 27 fibroblastoid colonies of 5 or more cells from a total of 200 culture dishes. Modderman et al. [54] reported that repeated exposure of plastic adherent bone marrow

cultures to the cytotoxin potassium thiocyanate selectively poisoned macrophages and other hematopoietic cell types, producing an enriched population of fibroblastoid cells. However, the authors failed to determine if the later contained a pool of progenitors capable of multi-lineage mesenchymal cell differentiation.

In the absence of a reliable purification scheme most laboratories continue to employ the method of plastic adherence to isolate MSCs, despite its limitations. Accordingly, the engraftment or therapeutic potential of MSCs has been evaluated in vivo by administering the plastic adherent cell fraction of bone marrow to experimental animals [55-57]. Since this population includes hematopoietic potential in vivo the aforementioned studies are confounded in that they do not provide a direct measure of the contribution made by MSCs to the experimental outcome.

Isolating a Homogeneous MSC Population from Umbilical Cord Blood (UCB) and Adult Bone Marrow (ABM)

Our lab has formulated a protocol based on immuno-depletion and positive selection of specific cell-surface markers to prepare primary cultures with a more homogeneous cell population of BM- and UCB-derived MSCs. Bone marrow and cord blood samples are enriched by negatively selecting for a variety of hematopoietic and monocyte cell surface markers with the use of density gradient cocktails and magnetic cell sorting. The samples are then positively selected for CD271, also known as LNGFR (low affinity nerve growth factor receptor), which has been shown to be a marker for the enrichment of nonhematopoietic stem cells. The cells are gown in supplemented media and when compared to MSCs isolated using the plastic adherence

method, the cells isolated with our protocol are shown, with the use of flow cytometry, to be a more homogeneous MSC population.

Purpose of the Present Study

The goal of this study was to compare the isolation and characterization of mesenchymal stem cells from two different sources, bone marrow and umbilical cord blood. Harvesting cells from bone marrow, the most common source, is an invasive procedure and it has been demonstrated that the number and the differentiating potential of bone marrow MSCs decreases with age [58]. Umbilical cord blood contains young, highly proliferative cells able to differentiate into the same lineages as MSCs derived from adult bone marrow.

A comparison of these two MSC sources was made by isolating samples from both UCB and BM using our novel isolation technique. We hypothesize that an immuno-depletion method against specific cell-surface markers will increase MSC homogeneity compared to the widely used plastic adherence method.

MATERIALS AND METHODS

Cells and cell culture

Isolation of mesenchymal stem cells from bone marrow

Bone marrow aspirates were obtained from the iliac crest of normal patients (41-65 years old) after informed consent and under a protocol approved by the VCU Institutional Review Board (VCU IRB# 3966). About 10-20 mL bone marrow aspirate were collected in a syringe containing heparin to prevent coagulation. The mononuclear cell fraction was isolated from red cells by density centrifugation over Histopaque 1077 (Sigma, St. Louis, MO). Magnetic-activated cell sorting (MACS) was performed removing hematopoietic and monocyte progenitor cells from the BM sample. CD 34^+ hematopoietic progenitor cells were magnetically labeled using MACS CD34 MicroBeads (Miltenyi Biotec, Auburn, CA). The magnetically labeled cells were removed by positive selection columns in the magnetic field of the MiniMACS Separator (Miltenyi Biotec, Auburn, CA). The same process was repeated with the use of a MACS Monocyte Isolation Kit which removed the monocyte lineage (CD3, CD7, CD14, CD56, and CD123) cells from the sample. The samples were then positively selected for CD271, also known as LNGFR (low affinity nerve growth factor receptor) with the use of a CD271 (LNGFR)-FITC and Anti-FITC MicroBeads MACS kit (Miltenyi Biotec, Auburn, CA). The cells were plated in expansion medium at a density of 10^5 cells/cm^2 in T_{25} tissue culture flasks (Nunclon, Roskilde, Denmark).

Isolation of mesenchymal stem cells from umbilical cord blood

Cord blood was collected into a sterile collection bag after informed consent of the mother using the guidelines approved by the VCU Institutional Review Board (VCU IRB# 2574). Blood was collected using a standard procedure with syringes containing L-heparin as anticoagulant. Mononuclear cells were separated from red cells by sedimentation with a solution of hydroxyethyl starch followed by density centrifugation over Histopaque 1077 (Sigma, St. Louis, MO). MSCs are enriched by depleting CD34+ , CD14+, CD3+, CD7+, CD16+, CD19+, CD56+, CD123+ and glycophorin+ cells using indirect MACS isolation kits as previously described for bone marrow (Miltenyi Biotech, Auburn, CA). The samples are then positively selected for CD271 with the use of a CD271 (LNGFR)-FITC and Anti-FITC MicroBeads MACS kit (Miltenyi Biotec, Auburn, CA). The cells were plated in expansion medium at a density of 10^5 cells/cm^2 in T_{25} tissue culture flasks (Nunclon, Roskilde, Denmark).

MSC Expansion Media

Expansion medium consists of Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY) containing 20% fetal bovine serum (FBS, Gibco), 0.4µl/ml gentamycin (Gibco), 2mM L-glutamine (Gibco), and 4ng/ml basic-fibroblast growth factor (β-FGF; Sigma). The immunodepleted cells were incubated in a humidified atmosphere at 37°C with 5%CO_2, and the medium was changed every 7 days until the

cells reached 80% confluence. Once adherent cells reached approximately 80% confluence, they were detached with Versene-EDTA (ethylenediaminetetraacetic acid; Cambrex, Walkersville, MD), washed with phosphate-buffered saline (PBS; Gibco, Grand Island, NY), centrifuged at 1000 rpm (82 x g) for 5 minutes, and replated at 1:3 in 3 x T_{75} tissue culture flasks under the same culture conditions. Cells were allowed to proliferate for 4-5 weeks in MSC expansion media and were tested for specific cell-surface markers at varying intervals throughout.

Flow Cytometry

After 10, 14, 28, and 42 days the cells were analyzed for the presence of specific cell surface markers. Cells were removed from tissue culture flasks adding Versene-EDTA (5mL per T_{75} flask) and incubating for 5 minutes at 37 °C. About $1x10^5$ cells/sample to be tested were removed and centrifuged for 5 minutes at 10,000 rpm. Supernatant was decanted and cells were resuspended in PBS solution. 1ml of $1x10^5$ cells/ml was transferred to a 12 x 75 glass disposable culture tube (Fischer Scientific). Glass tubes were then centrifuged at max speed (16,000 x g) for 2 minutes. PBS was decanted off and specific antibodies conjugated to either phycoerythrin (PE) or fluorescein isothiocyanate (FITC) were added sequentially. 10µl of IgG_1:FITC and 10µl of IgG_1:PE were added to each sample and run as isotype controls. CD105 and CD44 were used as positive MSC markers. 10µl of 105:PE and 10µl of 44:FITC were added. As a negative control, non-mesenchymal markers against CD45 and CD33 were used to test for hematopoietic cells. 10µl of 45:PE and 10µl of 33:FITC were added to

tubes. **Table 1** summarizes all information on antibodies used in the study. Tubes were quickly vortexed then incubated at 4 ℃ for 1 hour. After incubation, 1ml of PBS was added to each tube, tubes were vortexed, and spun for 2 minutes at max speed (14,100 x g) as a wash. The wash step was repeated and PBS decanted. 0.5ml of PBS was added to each tube, vortexed, and filtered through a 0.2 μm filter paper. Samples were put on ice and brought to the Flow and Imaging Cytometry Facility (Sanger Hall 2-012 Richmond, Virginia) to be analyzed.

Antibody	Primary host animal	Secondary antibody	Supplier
CD44	Mouse IgG2b anti-human	FITC-conjugated	BD Biosciences
CD105	Mouse IgG1 anti-human	Phycoerythrin-conjugated	R & D Systems
CD33	Mouse IgG1 anti-human	FITC-Conjugated	BD Biosciences
CD45	Mouse IgG1 anti-human	R-Phycoerythrin-conjugated	BD Biosciences
STRO-1	Mouse IgM anti-human	Anti-mouse IgM FITC-conjugated	R & D systems; Sigma
IgG_1 Isotype Control	Mouse	Anti-mouse IgG FITC-conjugated	R & D systems
IgG_1 Isotype Control	Mouse	Anti-mouse Phycoerythrin-conjugated	R & D systems
IgM Isotype Control	Mouse	Anti-mouse IgM FITC-conjugated	Sigma-Aldrich

RESULTS

Isolation and morphological analysis of ABM-derived and UCB-derived mesenchymal stem cells

It has been demonstrated that plastic adherent cultures elaborated from bone marrow and umbilical cord blood are an admixture of fibroblastoid and hematopoietic cell types, the later of which persist in the cultures even after serial passage [46, 52, 59]. Accordingly, we developed an isolation scheme to remove unwanted cells from the samples (**Figure 2**). The cells were subject to immuno-depletion using antibodies against CD3, CD7,CD14, CD16, CD19, CD34, and CD45, CD56, CD123, and glycophorin. This procedure removed essentially all hematopoietic and monocytic lineages from the cell fraction. Cells isolated from adult bone marrow and umbilical cord blood were grown in the same MSC expansion media consisting of DMEM supplemented with 20% fetal bovine serum, 0.4μl/ml gentamycin, 2mM L-glutamine, and 4ng/ml β-fibroblast growth factor (β-FGF). All isolated MSC populations displayed a spindle-shaped morphology (**Figure 3**). CB-derived MSCs had a heterogeneous morphology ranging from thin cells to flat round cells. However, upon contact with surrounding cells both BM-derived MSCs and UCB-derived MSCs changed into a homogeneous population and grew larger in size, with long protrusions at both poles (**Figure 3 b,d**).

The cell-surface antigen profile of immunodepleted cells was analyzed and compared with that of plastic adherence isolated cells. Flow activated cells sorting (FACS) analysis revealed that the immunodepleted cells from the positive/negative selection method uniformly expressed both CD44 and CD105 (99%) after 4 weeks of culture in MSC expansion media, but did not express CD33 or CD45 (HSC-specific markers). This is consistent with the findings for mesenchymal stem cells in the literature [18]. The cells from the plastic adherence method expressed variable levels CD44 and CD105 which is characteristic of a heterogeneous cell population (**Figure 4**).

A comparison between cells isolated from umbilical cord blood and adult bone marrow was made by looking at the expression of MSC cell-surface markers present after four and five weeks in MSC expansion media. The cell-surface antigen profile of UCB-derived MSCs was essentially the same as that of BM-derived MSCs with the bone marrow-derived MSCs expressing slightly higher levels of both MSC antigens (UCB-derived MSCs 90% doubly positive; ABM-derived MSCs 99% doubly positive) (**Figure 5, Table 2**).

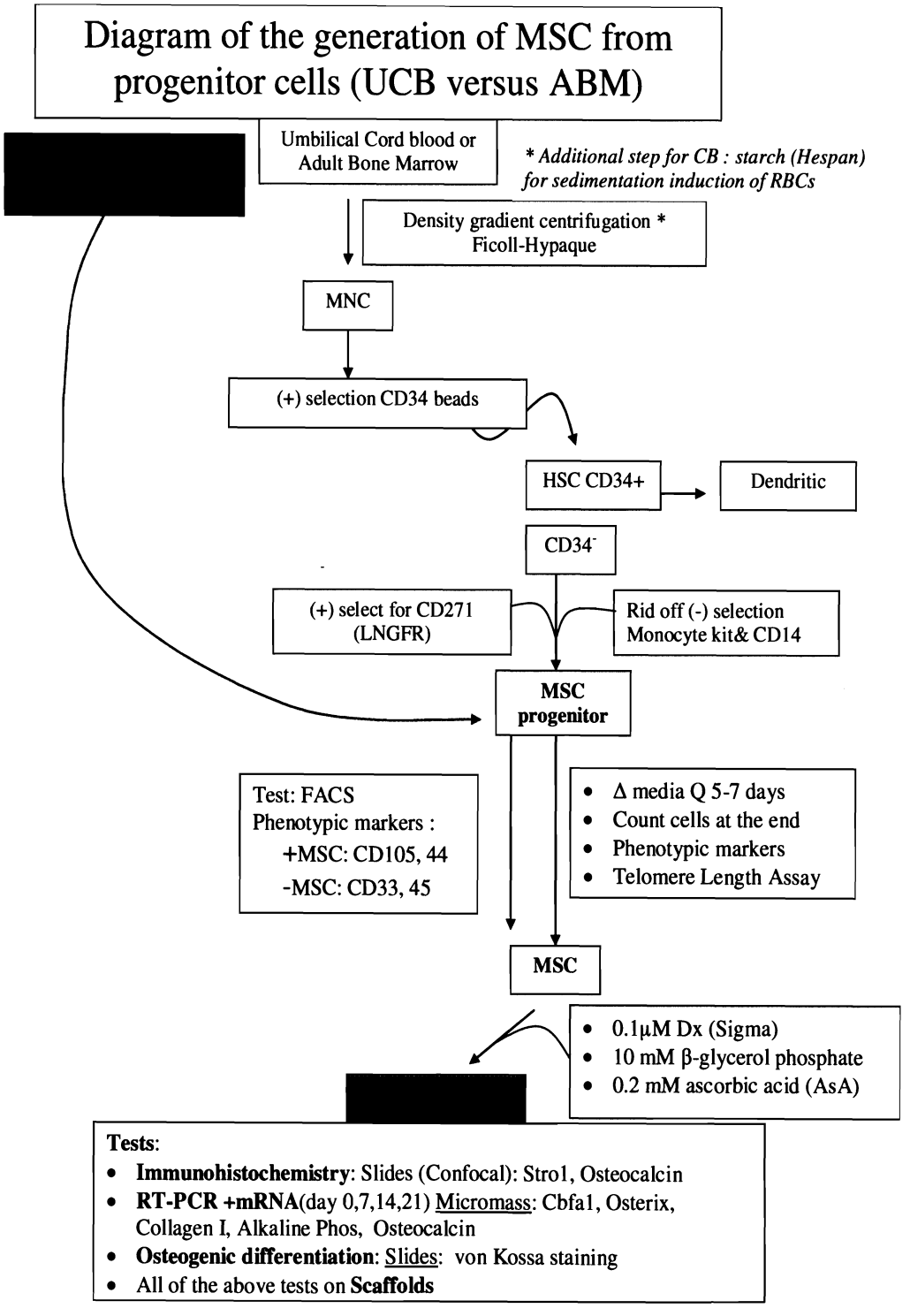
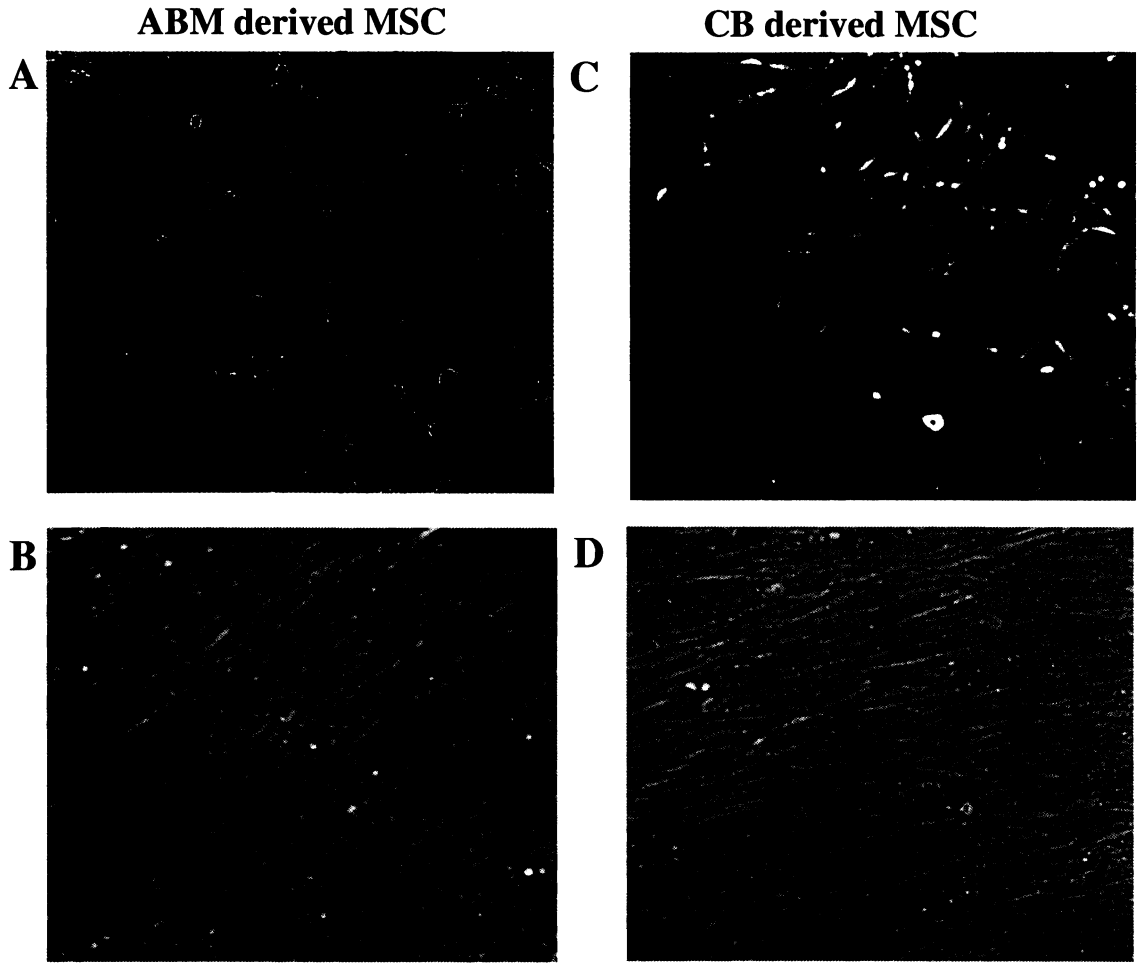
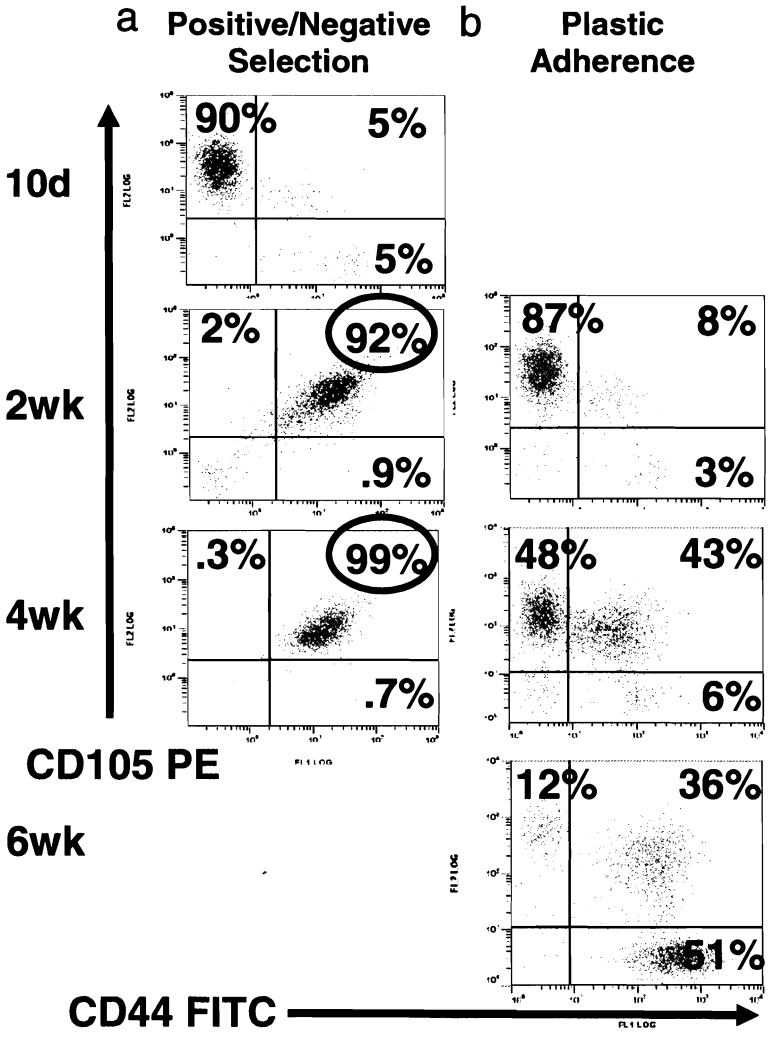
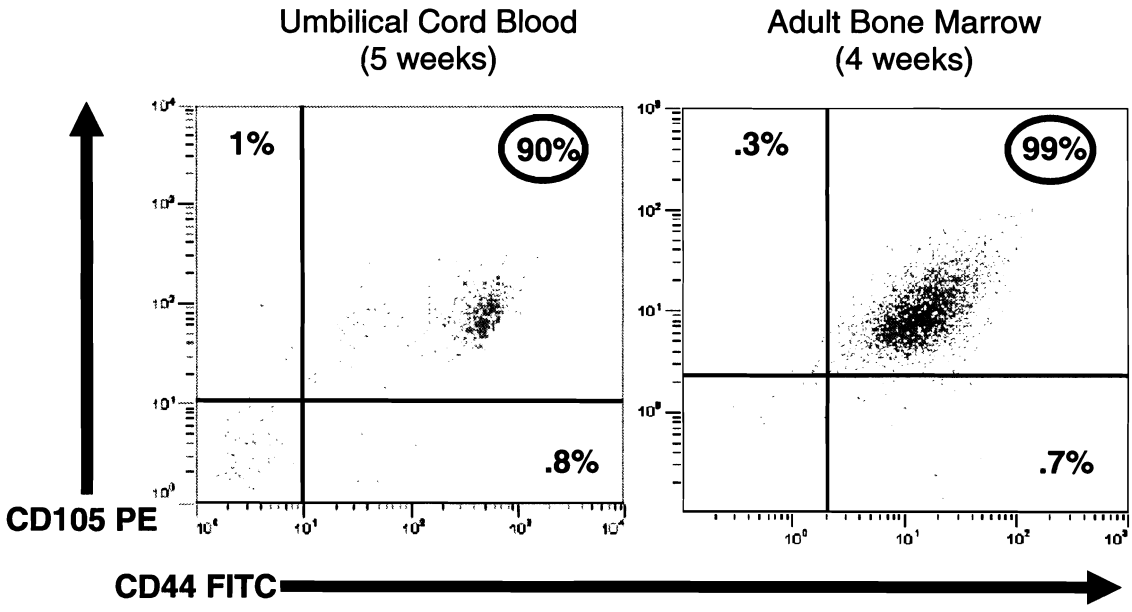


Diagram of the generation of MSC from progenitor cells (UCB versus ABM)
[redacted]
Umbilical Cord blood or Adult Bone Marrow
* Additional step for CB : starch (Hespan) for sedimentation induction of RBCs
Density gradient centrifugation * Ficoll-Hypaque
MNC
(+) selection CD34 beads
HSC CD34+
Dendritic
CD34-
(+) select for CD271 (LNGFR)
Rid off (-) selection Monocyte kit& CD14
MSC progenitor
Test: FACS
Phenotypic markers :
+MSC: CD105, 44
-MSC: CD33, 45
Δ media Q 5-7 days
Count cells at the end
Phenotypic markers
Telomere Length Assay
MSC
0.1μM Dx (Sigma)
10 mM β-glycerol phosphate
0.2 mM ascorbic acid (AsA)
[redacted]
Tests:
Immunohistochemistry: Slides (Confocal): Stro1, Osteocalcin
RT-PCR +mRNA(day 0,7,14,21) Micromass: Cbfa1, Osterix, Collagen I, Alkaline Phos, Osteocalcin
Osteogenic differentiation: Slides: von Kossa staining
All of the above tests on Scaffolds


ABM derived MSC
CB derived MSC
A
C
B
D


a
Positive/Negative Selection
b
Plastic Adherence
10d
2wk
4wk
6wk
90%
5%
5%
2%
92%
.9%
87%
8%
3%
.3%
99%
.7%
48%
43%
6%
12%
36%
51%
CD105 PE
CD44 FITC


Umbilical Cord Blood
(5 weeks)
Adult Bone Marrow
(4 weeks)
1%
90%
.8%
.3%
99%
.7%
CD105 PE
CD44 FITC

Cell Surface Marker	BM-MSC	UCB-MSC
CD33	—	—
CD45	—	—
CD44	+	+
CD105 (SH2)	+	+

DISCUSSION

A large body of evidence demonstrates that stromal tissue derived from adult bone marrow and umbilical cord blood contains clonogenic progenitor cells, some of which are considered to be multi-potent MSCs with the capacity to differentiate into a range of mesenchymal cell lineages including adipose tissue, bone, cartilage, tendon, and ligament [43, 60]. Despite considerable interest in the potential therapeutic applications of these cells, there is no well-defined protocol for the prospective isolation of human MSCs in order to properly study their biological properties prior to cell culture. Current methodologies for the isolation of MSCs are based upon those initially described by Friedenstein and colleagues, which rely upon the rapid adhesion of the stromal progenitor populations to tissue culture plastic and their subsequent rapid proliferation in vitro [18, 24, 45]. Such protocols result in a heterogeneous starting population of adherent bone marrow cells, of which only a minor proportion represent multipotent MSCs.

In this study we describe the isolation of a minor subpopulation of human stromal cells that represent a near homogeneous population of multipotent MSCs using an immuno-depletion isolation technique. This approach entailed removing the mononuclear cell fraction from red cells by density centrifugation followed by their separation from contaminating hematopoietic lineages using anti-CD14, anti-CD34, and anti-CD45 antibodies conjugated to paramagnetic beads. In order to identify that our immuno-depletion method resulted in a more homogeneous MSC population we

compared our method with the plastic adherence method. FACS analysis was run after 10 days of culture in MSC expansion media. The expression of CD105 was highly expressed (>90%) while CD44 was only present in a small percentage of cells (>5%). After 14 days the immunodepleted cell populations were >90% double positive for both the MSC-specific cell markers, CD44 and CD105, and as the culture progressed, the CD44 and CD105-positive cells gradually increased until week four where they were analyzed to be >99% double positive. After 4 weeks it is clear that the population isolated by immunodepletion is a more homogeneous MSC population. Cells cultured using the plastic adherence method were analyzed using the same MSC-specific markers. After 14 days, expression of one of the two markers was seen (87% CD105 positive; 3% CD44vpositive) and only 8% of the cells were double positive. After progressive culturing, the double positive cells gradually increased until less than half (35-45%) of the cells expressed both CD44 and CD105. This demonstrates that isolating MSCs using the plastic adherence method does isolate MSCs, but within the population other cell types must also be present. Tests for specific cell surface markers expressed on non-mesenchymal cells will help elucidate which other cell types comprise this heterogeneous population.

FACS analysis demonstrated that we were able to isolate a population of cells which expressed both MSC-specific markers. The morphology of the cells was similar in that they both expressed a spindle-shaped structure with numerous protrusions branching out. UCB-derived MSCs ranged from a flat, rounder shape to a thin, elongated appearance. As the cells proliferated out and came into contact with each

other, both ABM-derived and UCB-derived MSCs displayed a homogeneous fibroblastoid structure and became larger with long protrusions at both poles. These cells showed prolonged proliferative capacity without any morphological changes for more than 6 passages (over 3 months).

Future studies will entail a comparison of the telomere lengths between adult bone marrow-derived MSCs and umbilical cord blood-derived MSCs. When studying MSCs from umbilical cord blood one might assume the proliferation capacity of the young, healthy cells to be higher than those isolated from older adult bone marrow samples. Telomere length has been shown to be linked to proliferation capacity in that older, mature cells have shorter telomeres and lower proliferation rates. The telomere length between the two samples should differ in that the younger MSCs from cord blood should have significantly longer telomeres than those isolated from adult bone marrow. These young, highly multi-potent cells from cord blood could serve as a replacement for MSCs isolated from bone marrow. These cells might be less immunogenic in transplant hosts which would lead to a greater therapeutic potential of these cells. However, MSCs derived from UCB proliferate at least the same or even better than the ones from ABM, but the yield in extracting them still needs to be worked out.

MSCs hold promise for future stem-cell based therapy strategies and for tissue engineering. A major obstacle is the lack of definition and standardization of MSCs. Our study describes a reliable method to isolate a homogeneous population of MSCs from both adult bone marrow and umbilical cord blood and characterizes their

morphological and phenotypical characteristics. These cells can now be used to test their potential for stem cell-based therapies which will in turn contribute to establish a reliable quality control system for clinical applications.

CHAPTER 3

This chapter will focus on analyzing the potential of the MSCs isolated using our immuno-depletion method to differentiate into osteoblasts. We will look at the biology of bone, its remodeling capability, and osteogenesis. We hypothesize that MSCs isolated from both adult bone marrow and umbilical cord blood by the immuno-depletion method and cultured in osteogenic media will differentiate into functional osteoblasts.

CHAPTER 3

BIODEVELOPMENT OF OSTEOBLASTS FROM MESENCHYMAL STEM CELLS DERIVED FROM ADULT BONE MARROW AND UMBILICAL CORD BLOOD

ABSTRACT

In this study I set out to analyze the potential of our isolated MSCs to differentiate into functional osteoblasts. Mesenchymal stem cells were isolated from adult bone marrow and umbilical cord blood using the process of immunodepletion as described in the previous chapter. Cells from each sample were plated on 6-well plates and induced down the osteogenic pathway by culturing in osteogenic media (10mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 0.2mM ascorbic acid). Time intervals were set up so after 3 weeks, cells from each ABM or UCB sample had been in osteogenic media for either 0, 7, 14, or 21 days. These time points were selected to represent progenitor pools of early mesenchymal, osteo-progenitor, and mature cell stages of osteoblast differentiation.

The results showed that after osteoinduction, cells from both ABM-derived and UCB-derived MSCs deposited a calcified, mineralized extracellular matrix as was seen by von Kossa staining. With the use of confocal microscopy the transition of MSCs into osteoblasts was visualized with fluorescent markers specific for both MSC progenitor and osteoblast cells. The gene expressions of the cells was also looked at

which confirmed the presence of the osteo-specific molecular markers: Cbfa-1, osterix, osteocalcin, alkaline phosphatase, and collagen type-I.

INTRODUCTION

Brief insights in bone biology

Bone tissue in the adult skeleton is arranged in two architectural forms: trabecular, also called cancelous or spongy bone (around 20% of the total skeleton), and cortical or compact bone (around 80% of the total skeleton) [61].

The proportions of these two architectural forms differ at various locations in the skeleton. Cortical bone is almost solid, being only 10% porous, and can be divided into different subgroups: long bones (femur and tibia), short bones (wrist and ankle), and flat bones (skull vault and irregular bones) [61]. On the other end, trabecular bone presents a higher porosity, 50-90%, making its modulus and ultimate compressive strength around 20 times inferior than that of cortical bone [61]. Trabecular bone is arranged in a sponge-like form, with a honeycomb of branching bars, plates and rods of various sizes called trabeculae. It is commonly found in the metaphysis of long bones, covered by cortical bone, and in the vertebral bodies.

The elaboration, maintenance, and resorption of this remarkable tissue results from the interaction of three cell types: osteoblasts, osteocytes, and osteoclasts. Remodeling results from the action of cells that form bone, osteoblasts, and cells that resorb bone, osteoclasts. Osteoblasts produce an extracellular matrix, osteoid, which is composed primarily of type I collagen, the balance being non-collagenous proteins. This matrix is calcified by osteoblasts and during this process the cells become encased

in lacunae within the calcified material and become osteocytes, cells which help to maintain the structure of the bone. Upon completion of bone formation, a layer of resting osteoblasts remains on the surface of the bone which is covered by non-calcified osteoid. Renewal of the osteoblast population results from the differentiation of osteoprogenitor cells residing in the bone marrow compartment. Defects such as microfractures are repaired by the coupled action of osteoclasts and osteoblasts.

The overall integrity of bone appears to be controlled by hormones, such as estrogen and testosterone, and many other proteins secreted by both hematopoietic bone marrow cells and bone cells. Certain of these cytokines, i.e., growth factors, the interleukins (interleukin-1, interleukin-6, and interleukin-11), transforming growth factor-β and tumor necrosis factor-α, play a significant role in controlling the activities of the osteoblasts and osteoclasts. During the post-menopausal years, women suffer from accelerated bone loss which can ultimately lead to osteoporosis, a debilitating disease that affects 20 million women in the United States [39]. The accelerated bone loss coincides with estrogen withdrawal and an apparent enhancement of the secretion of cytokines which accelerates osteoclast activity. The increased osteoclast resorption of bone is coupled to an increased bone formation rate, but the rate of formation lags behind resorption. The net result is trabecular bone loss and weakening of the bone structure. Continued bone loss leads to heightened risk of fractures of the vertebrae and hip. Eventually, the rapid bone turnover ceases, leaving few osteoblasts to form new

bone. The loss of osteoblasts may be related to a decline in the renewing osteoprogenitor cell population.

Bone development involves a series of different stages that comprise the proliferation of osteoprogenitor cells at the outset and the differentiation of progenitors into osteoblasts. The major products of osteoblasts are deposited extracellularly, which makes for clear visualization of the differentiation process. The extracellular matrix of bone is calcified and collagenous. Bone-type collagen – type I, designated ($\alpha 1(I)_2\alpha_2$) – is prevalent in several major connective tissues and comprises 85 – 90% of total organic bone matrix [62].

Osteoblast precursor cells

A series of experiments have been performed that demonstrate the existence of progenitor cells that give rise to bone, cartilage, muscle, tendon, fat, and a mature stromal phenotype that supports hematopoietic differentiation. These cells are referred to as Mesenchymal Stem Cells (MSCs). We have demonstrated a refined technique for the isolation and extensive subcultivation of human MSCs by the process of immunodepletion. Purified MSCs have been characterized with respect to their complement of cell surface and extracellular matrix molecules as well as their secretory cytokine profile in control and experimental conditions [42]. A series of monoclonal probes which react with the surface of human MSCs both in situ and in vitro have been developed. The murine IgM monoclonal antibody (Ab) STRO-1, produced from an immunization with a population of human $CD34^+$ bone marrow cells, can identify a cell

surface antigen by stromal elements in human bone marrow. A STRO-1^+ subset of cells is capable of differentiating into multiple mesenchymal lineages including adipocytes, osteoblasts, and chondrocytes. STRO-1 is a valuable Ab for the identification, isolation, and functional characterization of human mesenchymal stem cells.

Molecular regulation of osteogenic differentiation

The induction of MSC osteogenesis is a highly programmed process when illustrated *in vitro*. Treatment with the synthetic glucocorticoid dexamethasone (Dex) stimulates MSC proliferation and supports osteogenic lineage differentiation [63, 64]. Dex promotes differentiation and mineralization by inducing the expression of the main transcription factors. Organic phosphates, such as β-glycerol phosphate (βGP), also support osteogenesis by playing a role in the mineralization and modulation of osteoblast activities [65, 66]. Free phosphates can induce the messengerRNA (mRNA) and protein expression of osteogenic markers such as osteocalcin, and these phosphates have known effects on the production and nuclear export of key osteogenic transcription factors. Other supplements, such as ascorbic acid phosphate (vitamin C) and 1,25-dihydroxyvitamin D_3, are used for osteogenic induction as they are involved in increasing alkaline phosphatase activity in osteogenic cultures and promoting the production of osteocalcin [67].

Osteoblast-specific transcription factors

The differentiation of precursor cells into osteoblasts is controlled by a specific transcription factor, the core binding factor alpha-1 (Cbfa-1). This member of the runx/cbfa family of transcription factors was first identified as the nuclear protein binding to an osteoblast-specific cis-acting element activating the expression of *osteocalcin*, the most osteoblast-specific gene. Most of the described osteoblast genes contain binding sites for the product of the *Cbfa-1* gene in their promoter regions and are thus up regulated upon Cbfa-1 induction [68-70]. Consistent with this ability, genetic experiments identified Cbfa-1 as a key regulator of osteoblast differentiation in vivo. Indeed, analysis of Cbfa-1-deficient mice revealed that osteoblast differentiation is arrested in absence of Cbfa-1, demonstrating both that it is required for this process and that no parallel pathway can overcome its absence [71]. Cbfa-1 was found to be a critical gene not only for osteoblast differentiation, but also for osteoblast function.

Osterix (Osx), a zinc-finger-containing protein, was shown to be the second osteoblast-specific transcription factor. Osx-deficient mice lack osteoblasts and do not form endochondral or intramembranous bones [72]. It is likely that Cbfa-1 and Osx control different stages of osteoblast differentiation and, as Osx is not expressed in Cbfa-1-null mice but Cbfa-1 is expressed normally in Osx-deficient mice, it has been suggested that Osx acts downstream of Cbfa-1 [73, 74].

Purpose of Present Study

The goal of this study was to analyze the potential of our isolated MSCs to differentiate into functional osteoblasts. Mesenchymal stem cells were isolated from adult bone marrow and umbilical cord blood using the process of immuno-depletion as described in the previous chapter. Cells from each sample were plated on 6-well plates and induced down the osteogenic pathway by culturing in osteogenic media (10mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 0.2mM ascorbic acid). Time intervals were set up so after 3 weeks, cells from each ABM or UCB sample had been in osteogenic media for either 0, 7, 14, or 21 days. These time points were selected to represent progenitor pools of early mesenchymal, osteo-progenitor, and mature cell stages of osteoblast differentiation. Once the time course was complete, experiments were run to test the differentiation potential of both adult bone marrow and umbilical cord blood MSCs into osteoblasts. Confocal microscopy was employed to visualize the ratio of specific markers for both MSCs and mature osteoblasts. With the use of von Kossa staining the deposition of a calcified matrix on the surface of cells was evident. MessengerRNA was also isolated from the cells to check for the presence of specific osteoblast marker genes by real time-RT-PCR.

MATERIALS AND METHODS

Culture and propagation of MSCs in the undifferentiated state

MSCs were maintained in T_{75} tissue culture flasks in appropriate MSC culture media (DMEM, 20% FBS, 0.4µl/ml gentamycin, 2mM L-glutamine, and 4ng/ml β-FGF). After four passages (four weeks), the MSCs were replated at 2 x 10^5 per well on normal 6-well plates (Nunc Inc., Roskilde, Denmark) for experimental analysis. Four 6-well plates for each bone marrow and umbilical cord sample were prepared. In each of the 6-well plates, three of the wells contained sterile square coverslips while the three remaining wells remained empty. The cells were incubated in a humidified atmosphere at 37°C with 5%CO_2, and the media was changed every 4-5 days until cells reached 90% confluency.

Stimulation of osteogenic differentiation

To induce osteogenic differentiation, cells were cultured in MSC culture media supplemented with 10mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 0.2mM ascorbic acid (Sigma-Aldrich, St. Louis, MO). Osteogenic media was changed every 4-5 days. Time intervals were set up so after 3 weeks, cells from each BM or CB sample have been in osteogenic media for either 0, 7, 14, or 21 days.

Von Kossa Staining

A cover slip from each of the time periods was removed and rinsed with phosphate buffered solution (PBS). The cells were fixed with 10% formalin solution for one minute then rinsed with PBS. Silver nitrate solution (5%) was added and the cells were exposed to UV light for 20 minutes. The coverslips were washed in distilled water three times. Sodium thiosulfate (5%) was added for 5 minutes then again washed three times with distilled water. The coverslips were then dehydrated, cleared, and mounted on a slide with proper mounting solution. Images were taken using a light microscope under a 10x magnification.

Immunohistochemistry

Coverslips (one from each time period) were removed from the 6-well plates and rinsed with phosphate-buffered saline (PBS). Small wells were created on each cover slip by using an Immedge Pen (Vector Laboratories, Burlingame, CA). Cells were fixed with ice cold methanol and then rinsed twice with TBS. Cells were permeabilized and blocked by adding a solution of 0.1% Triton X-100 and 3% normal goat serum (Vector Laboratories, Burlingame, CA) in TBS and then washed twice with TBS. Slides were then incubated with primary antibody for one hour. A mouse monoclonal anti-human STRO-1 antibody (R&D Systems, Minneapolis, MN) and a rabbit polyclonal antibody to osteocalcin (R&D Systems, Minneapolis, MN) were used at a 1:50 dilutions. The coverslips were washed five times with PBS and the following fluorophore-conjugated secondary antibodies were incubated for one hour at a dilution of 1:200 with either chicken anti-rabbit Alexa Fluor 488 (green) or goat anti-mouse IgM

Alexa Fluor 594 (red) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), as specified in respective figure legends. Coverslips were washed five times with PBS. A fluorescent DNA binding stain (Hoechst 34580; Molecular Probes, Eugene, OR) was used at a concentration of 5μg/ml and incubated for 30 minutes to visualize nuclei. The coverslips were then washed five times with PBS. Coverslips were applied to a slide with an anti-fading medium (Vectashield; Vector Laboratories, Burlingame, CA). Slides were viewed and images stored using a laser scanning confocal microscope (model LSM 520 Meta; Carl Zeiss Inc, Thornwood, NY). Each sample was excited using a 405nm, a 488nm, and a 543nm laser. The cells were excited separately by each laser to eliminate any possible cross-excitation. All images were taken with an optical planar slice of 1μm thickness using a 64x oil-immersion objective. A composite image was made by combining the images from the three detection filters. In addition, negative controls were stained with a secondary antibody only and used to verify the staining specificity of binding of the secondary antibodies.

Isolation of RNA and DNA Removal

On day 21, total RNA from each time point was extracted with 1mL of TRI-Reagent (Invitrogen) per well according to the manufacturers instructions. In brief, after washing cells with sterile PBS, RNA was extracted with TRI-Reagent/chloroform solution and precipitated with isopropanol. The RNA pellet was washed twice with 75% alcohol before re-dissolving the RNA in RNase-free diethyl pyrocarbonate (DEPC) water. The RNA sample was cleaned using MessageClean Kit (Nashville,

TN), which removed DNA contaminations from RNA. RNA was quantified by spectrophotometry using BioRad, SmartSpec 3000 (Hercules, CA). Purified RNA samples from each time period were sent for Real Time-RT-PCR analysis.

Real Time Reverse Transcriptase Polymerase Chain Reaction Analysis

RNA samples were sent to the MCV-VCU Nucleic Acids Research facility to analyze the expression of specific osteoblast genes. Primer sequences were designed at the Nucleic Acids Core Lab and were analyzed for their specificity at the National Center for Biotechnology Information (NCBI). The genes analyzed were: Cbfa-1, Osterix, Osteocalcin, Alkaline phosphatase, and Collagen type I. 18s rRNA was used as an endogenous control.

RESULTS

Immunohistochemical Staining

Immunohistochemical staining was used to detect the presence of mesenchymal stem cell and osteogenic lineages after 0, 7, 14, and 21 days in osteogenic medium. By looking at markers highly expressed by MSCs (STRO-1) and mature osteoblasts (Osteocalcin) we were able to follow the cells through the differentiation process into maturing osteoblasts. Osteocalcin is a protein unique to bone cells that causes the proper deposition of phosphate and calcium on the organic collagen matrix during new bone formation. The results showed that there was expression of each antibody at varying levels throughout the differentiation time course. As seen in **Figure 6**, STRO-1 positive cells (red fluorescence) are expressed in Day 0 and Day 7 bone marrow cells. By Day 14 however, the intensity of the MSC STRO-1 marker greatly diminishes. The osteocalcin antibody is minimally present in Day 0 bone marrow cells, but after induction in osteogenic media the cells begin producing the osteocalcin protein. After 14 days we see very strong staining for osteocalcin and very minimal STRO-1 presence. As the cells differentiate in the osteogenic media there is a noticeable morphological change. The cells transition from long spindle-shaped cells (Day 0) to more clustered cuboidal shaped cells (Day 14). **Figure 7** shows another bone marrow sample stained with STRO-1 and osteocalcin antibodies. Again we see the presence of STRO-1 after 7 days in osteogenic media slowly decreasing until Day 21 where almost no STRO-1 is

present. The osteocalcin fluor shifts in the opposite direction by gradually getting stronger in intensity as the time in culture progresses. **Figure 8** illustrates the osteogenic differentiation of umbilical cord blood-derived MSCs. At Day 0 the cells are unquestionably MSCs in a multi-potent state because the STRO-1 Ab is highly expressed while little to no osteocalcin is seen. Then, as osteoinduction media is introduced, the cells begin the differentiation process. The cells noticeably change their shape by bunching together into the distinctive cuboidal shape and start producing high levels of the osteocalcin protein (**Fig. 8 - Day 14, 21**).

These images (**Figs. 6-8**) illustrate that our immunodepletion technique allows for isolation and expansion of mesenchymal stem cells (MSCs) from adult bone marrow and umbilical cord blood by the strong presence of the MSC marker STRO-1. They also demonstrate the ability of these multi-potential MSCs to be differentiated *in vitro* into mature, bone producing osteoblast cells after 3 weeks in osteogenic media.

Detection of osteogenic marker expression by RT-PCR analysis

To confirm that the cells were indeed committed to the osteogenic lineage, RT-PCR was used to detect the expression of prominent osteo-specific molecular markers: Cbfa-1, osterix, osteocalcin, alkaline phosphatase, and collagen type-I. MessengerRNA was isolated from both adult bone marrow and umbilical cord blood after their respective time period in osteogenic media, rid of any DNA contamination, and sent to the Nucleic Acid Research Facility to be analyzed. Results were normalized to an 18s rRNA endogenous control for each sample.

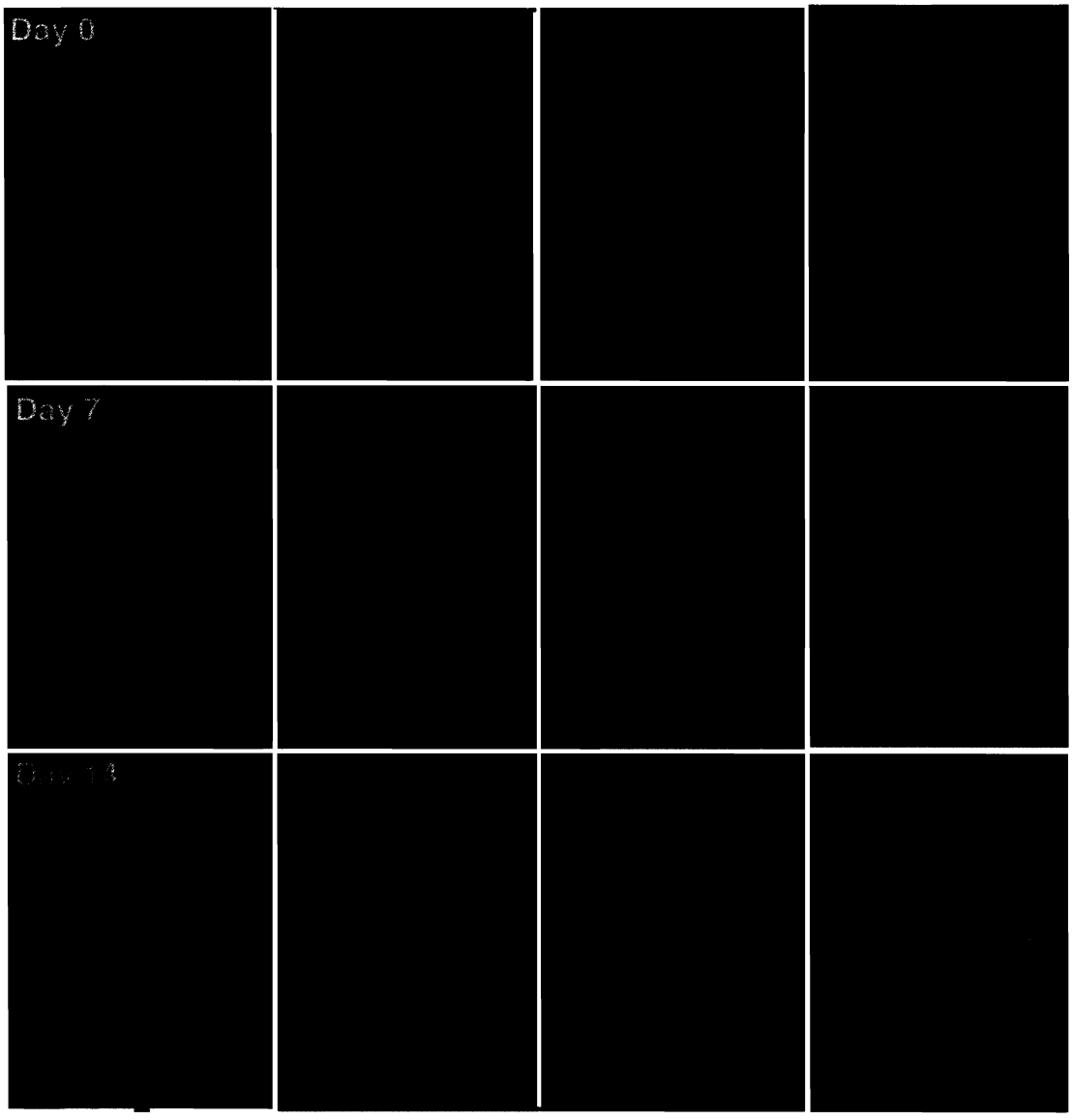
The expression of osteogenic markers from bone marrow cells is depicted in **Figure 9**. The main transcription factor, Cbfa-1, was highly expressed early in the differentiation process and its levels remained constant throughout the 3 weeks in osteogenic culture, while osterix, the transcription factor thought to act downstream of Cbfa-1, maintains low levels until 14 to 21 days in osteogenic media where its transcription more than doubles. Osteocalcin levels are present but do not fluctuate greatly throughout the differentiation process. Alkaline phosphatase and collagen type I levels both gradually increase over the 3 week differentiation process as more cells are able to express their genes as they become mature, fully functional osteoblast cells.

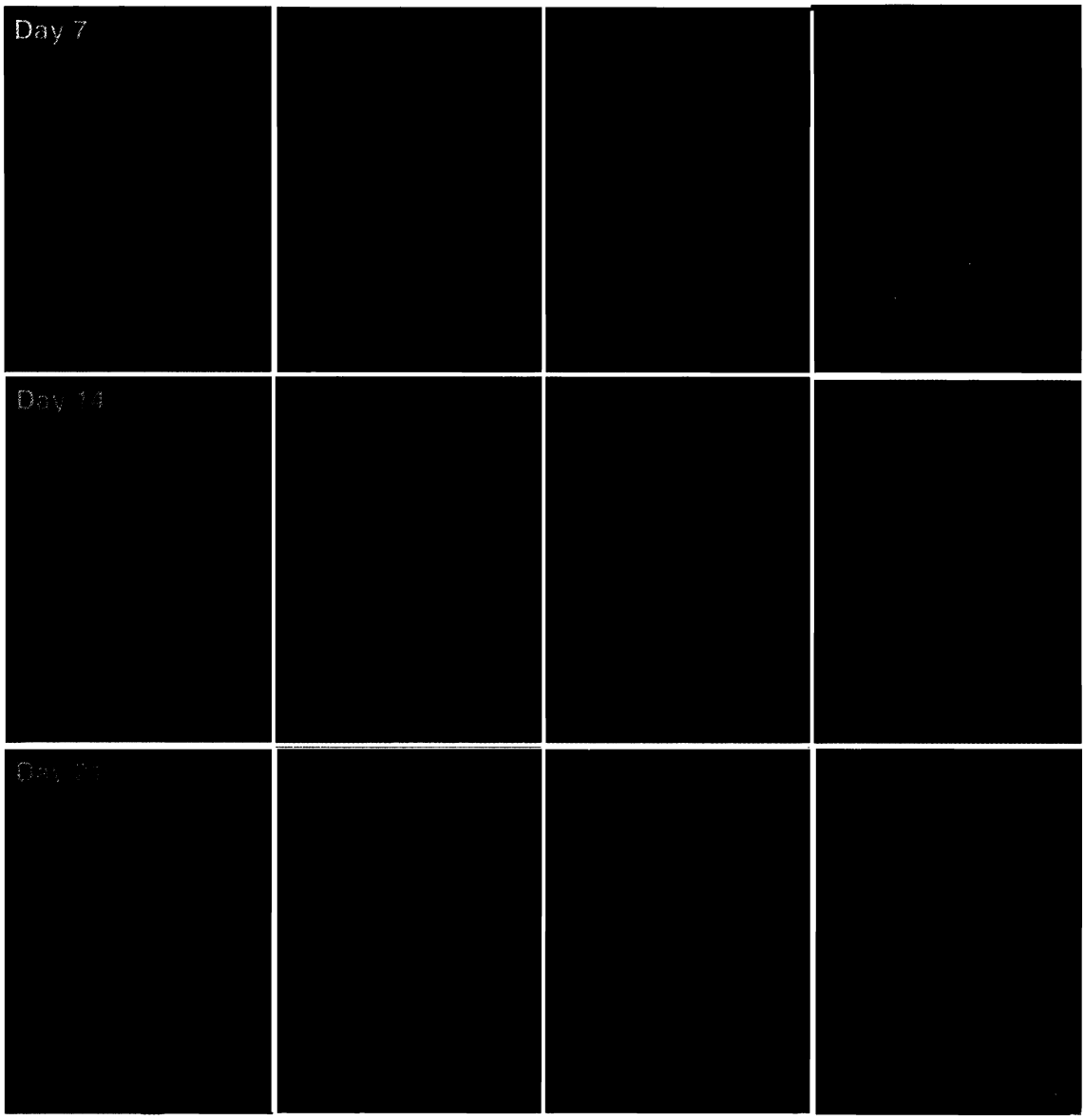
Osteogenic markers expressed by MSCs isolated from umbilical cord blood are illustrated in **Figure 10**. Cbfa-1, the main transcription factor, is present but not in such a high level as the bone marrow cells. Osterix was undetectable on Days 0 and 7 but Day 14 the late acting transcription factor started to be expressed. After 21 days in osteogenic media the UCB-derived cells produced an extremely high level of osterix. Osteocalcin, alkaline phosphatase, and collagen type I were all present, but their levels did not significantly fluctuate throughout the differentiation process.

Osteogenic differentiation of ABM-derived and UCB-derived MSCs

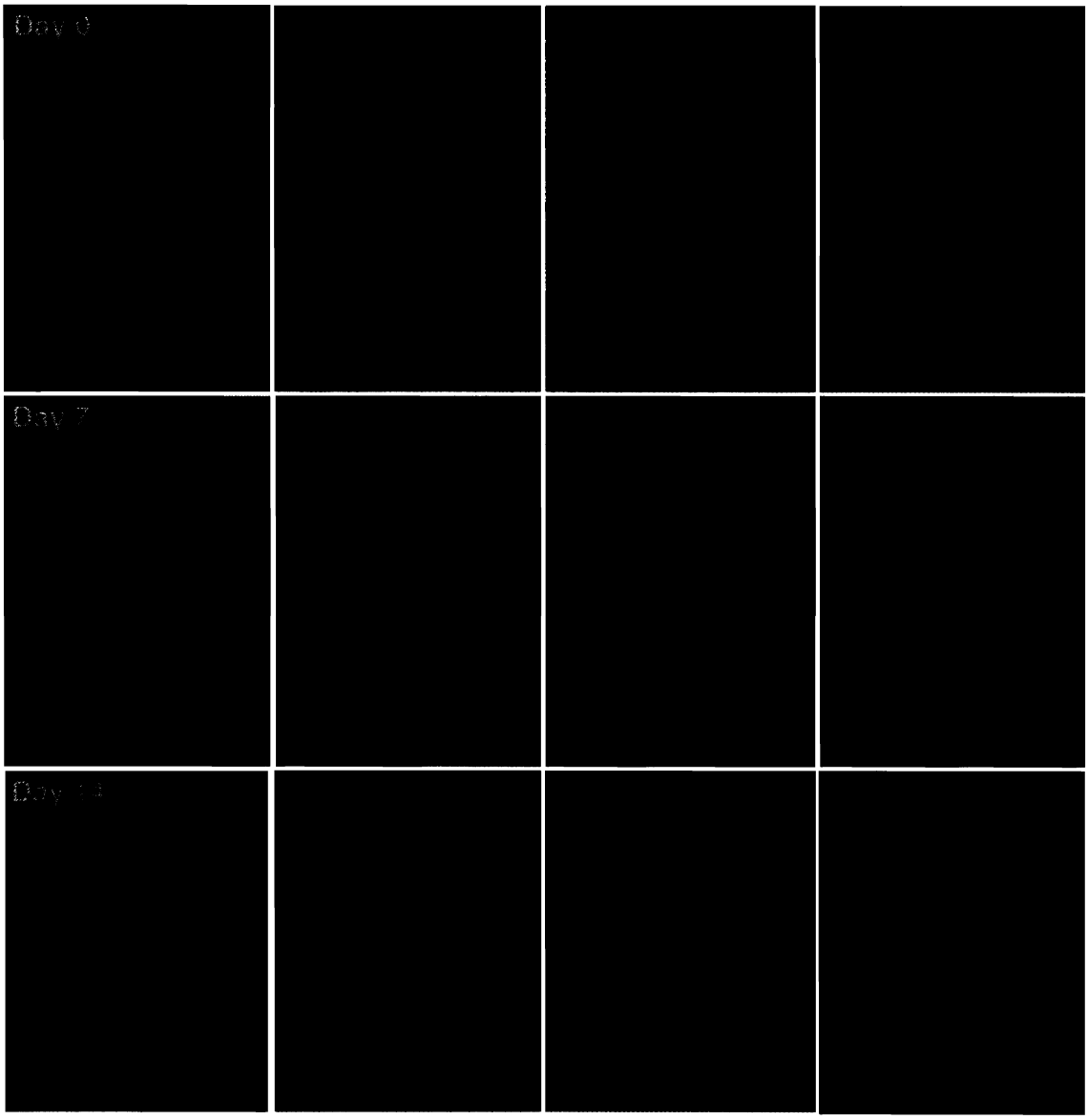
During the 21 day assay period, MSCs cultured with osteogenic media (10mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 0.2mM ascorbic acid) underwent a dramatic change in cellular morphology which was accompanied by a significant increase in calcification and mineralization. Matrix production occurred as early as 7

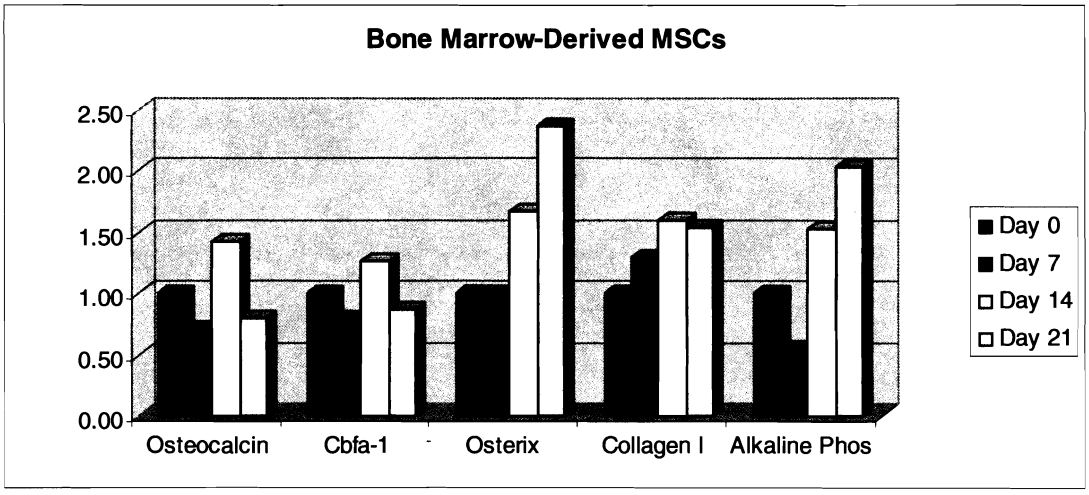
days after induction and increased with time in culture. After 14 and 21 days of the culture period, the matrix was mineralizing and the deposition of calcium became evident by von Kossa staining (**Figure 11**). No calcification was seen in control cultures without osteogenic media (**Fig. 11 a,d**). MSCs cultured in osteogenic media showed a change in their morphology from spindle-shaped to cuboidal in as little as 7 days (data not shown). While control cultures grew as a uniform sheet of cells, osteogenic cultures began to form multilayered nodular structures as the apparent result of coalescing cellular aggregates (**Fig. 11 b,e**). At day 21, osteogenic cultures contained a well-developed uniform sheet of bone-like material which stained heavily for the presence of calcification (**Fig. 11 c,f**).

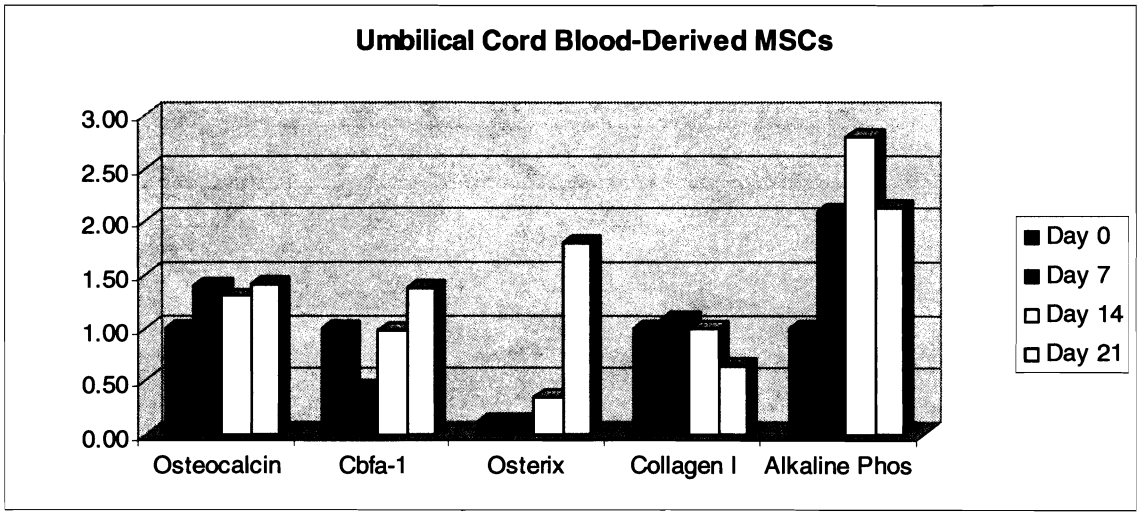

Day 0
Day 7
Nuclei
Red: Stro-1
Green: Osteocalcin
Composite

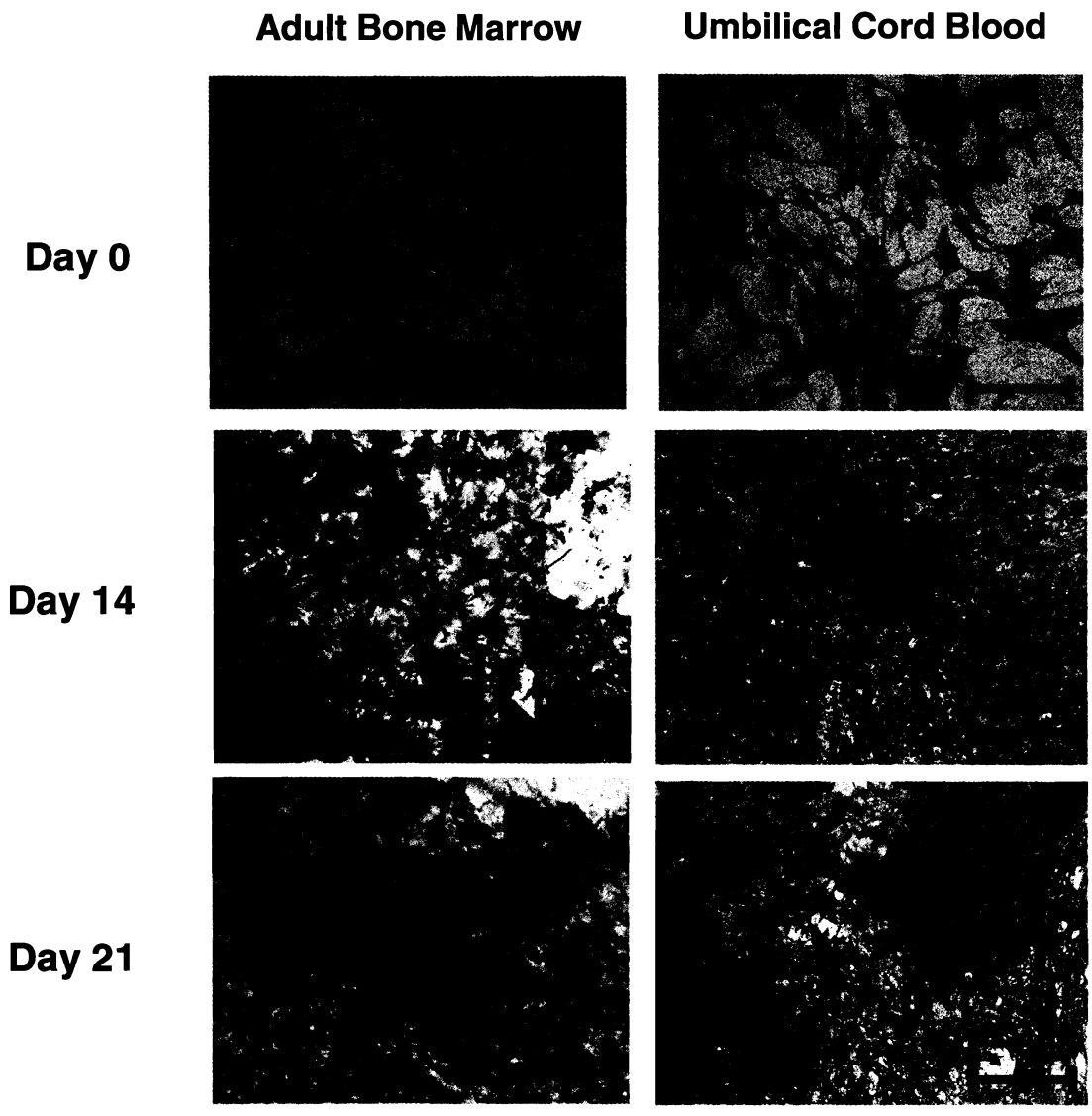


Nuclei **Red: Stro-1** **Green: Osteocalcin** **Composite**


Day 0
Day 7
Day 14
Nuclei
Red: Stro-1
Green: Osteocalcin
Composite


Bone Marrow-Derived MSCs
2.50
2.00
1.50
1.00
0.50
0.00
Osteocalcin
Cbfa-1
Osterix
Collagen I
Alkaline Phos
Day 0
Day 7
Day 14
Day 21


Umbilical Cord Blood-Derived MSCs
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Osteocalcin
Cbfa-1
Osterix
Collagen I
Alkaline Phos
Day 0
Day 7
Day 14
Day 21


Adult Bone Marrow
Umbilical Cord Blood
Day 0
Day 14
Day 21

DISCUSSION

This study demonstrates that purified, culture-expanded human MSCs can be directed into the osteogenic lineage in vitro, culminating in mineralized matrix production, and thereby establishing a system for studying human osteoblast differentiation from mesenchymal stem cells. The addition of osteogenic supplements (10mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 0.2mM ascorbic acid) to MSC cultures was capable of inducing rapid osteogenesis as defined by the appearance of osteoblastic cell morphology, the formation of a mineralized, calcified extracellular matrix, reactivity with anti-osteogenic cell surface monoclonal antibodies, and the production of osteo-specific genes.

Cultured human MSCs provide a useful model for evaluating the multiple factors responsible for the step-wise progression of cells from undifferentiated precursors to secretory osteoblasts, and eventually terminally differentiated osteocytes. While primary cultures of human MSCs appear morphologically similar to bone marrow stroma-derived cells, the precise conditions for the cultivation of these cell types have a profound effect on cell selection and behavior. The cell seeding density, type of tissue culture plastic, and source of fetal calf serum are known to affect the developmental potential of cultured cells [42, 75]. We maintained cells in the log phase of growth without allowing them to become confluent and form multilayers, since differentiation of mesenchymal stem cells is know to be triggered by increasing cell density [76]. Our protocol for handling human MSCs supports the retention of stem

cell-like properties for many passages, or until such time that the cells are placed in an inductive environment.

In this study we demonstrate the capability to differentiate MSCs isolated from adult bone marrow and umbilical cord blood into mature osteoblasts. Setting up a time course in which the cells were in osteogenic media for different time periods allowed us to visualize the differentiation stages.

The murine IgM monoclonal Ab STRO-1 can identify a cell surface antigen by stromal elements on non-hematopoietic cells. A STRO-1^+ subset of cells is capable of differentiating into multiple mesenchymal lineages including adipocytes, osteoblasts, and chondrocytes. STRO-1 is a valuable Ab for the identification, isolation, and functional characterization of human mesenchymal stem cells. Osteocalcin is a 5800 MW extrahepatic vitamin K dependent protein uniquely expressed by osteoblasts. Osteocalcin is one of the most abundant proteins in the body and the second most abundant protein in the skeletal system next to collagen. Osteocalcin functions in facilitating the binding of calcium ions and hydroxyapatite together to be deposited on the collagen matrix of developing bones. Osteocalcin is a highly conserved 46-50 amino acid single chain protein that contains three vitamin K-dependant gamma-carboxyglutamic acid residues. The antibody was a polyclonal antibody raised against the full chain of human osteocalcin. With the use of confocal microscopy we were able to detect the presence of the mesenchymal stem cell marker (STRO-1) and osteogenic marker (osteocalcin) after 0, 7, 14, and 21 days in osteogenic medium. This experiment reconfirmed that we had a homogeneous MSC population able to retain stem cell-like

properties for many passages by the strong presence of the STRO-1 antibody on Day 0 samples. The images detail the transition of MSC progenitor cells into osteoblasts by characterizing the down regulation of the MSC marker STRO-1 and the up regulation of the osteoblast protein, osteocalcin. Both ABM-derived MSCs and UCB-derived MSCs were shown to be induced into osteoblasts. Both cell sources expressed high levels of the osteocalcin marker and displayed distinct morphological changes after 3 weeks in osteogenic media.

Using RT-PCR we were able to further demonstrate that the cells were indeed committed to the osteogenic lineage. The expression of the prominent osteo-specific molecular markers: Cbfa-1, osterix, osteocalcin, alkaline phosphatase, and collagen type-I were detected throughout the differentiation process. Although both bone marrow and umbilical cord blood produced significant levels of each marker, the adult bone marrow cells expressed much more compared to their 18s endogenous controls. This variation is due to the fact that both adult bone marrow and umbilical cord blood samples were compared to standards from their respected sources.

Staining for calcification using the von Kossa method revealed the deposition of calcium crystals by the osteoblast cells (Day 14, 21). The MSCs in osteogenic culture formed a continuous interconnected network of mineralized extracellular matrix. The sheet-like uniformity of this osteogenic response is reminiscent of the intramembranous ossification which occurs in developing calvariae and long bone diaphyses [77]. The observation that virtually all cells are involved in the formation of this bone-like material argues against the selective induction of a limited number of osteoprogenitors

and supports the characterization of this population of cells as homogeneous. The MSCs which were maintained in MSC expansion media did not express any signs of calcification. The morphological changes noticeable during the differentiation process are also of importance. Cells at Day 0 maintained the typical MSC spindle-shaped morphology while cells induced in osteogenic media clustered together forming bone nodule-like structures. Both MSCs from ABM and UCB were capable of differentiating into calcium depositing osteoblast cells.

The isolation of human MSCs and their cultivation described in this study provide a system for analyzing the events of MSC commitment and osteogenic differentiation into fully functional secretory osteoblasts and osteocytes. After 3 weeks in osteogenic media containing 10mM β-glycerol phosphate, 10^{-7} M dexamethasone, and 0.2mM ascorbic acid, cells isolated from bone marrow and umbilical cord blood were shown to be fully differentiated, mature osteoblasts. With the ability to examine the cell and molecular events of differentiation from purified, culture-expanded multipotent MSCs, we have the capacity to address experimental questions which cannot be answered using more mature and heterogeneous human osteoblasts.

GENERAL CONCLUSIONS

The data from the two studies presented in this thesis demonstrate that we were able to isolate a homogeneous population of multipotent MSCs using our immunodepletion technique. Previous studies with MSCs have isolated cells based on plastic adherence, which consisted of a heterogeneous population of cells from multiple lineages. Our method of removing the unwanted cells from the bone marrow or umbilical cord sample allows for the characterization and therapeutic potential of these MSCs to be studied without other cell types affecting the results. We also demonstrated the possibility of MSCs from two different sources to differentiate into mature, functional osteoblasts. This study confirmed that our starting populations of cells were indeed multi-potential MSCs capable of being osteoinduced.

MSCs present an exciting progenitor cell source for applications of tissue engineering and regenerative medicine. Future modalities may include direct implantation and/or *ex vivo* tissue engineering, in combination with biocompatible/biomimetic biomaterials and/or natural or recombinantly derived biologics. MSCs may also be considered for gene therapy applications for the delivery of genes or gene products. Another intriguing prospect for the future is the use of MSCs to create "off-the-shelf" tissue banks. To fully harness the potential of these cells, future studies should be directed to ascertain their cellular and molecular characteristics for optimal identification, isolation, and expansion, and to understand the natural, endogenous roles of MSCs in normal and abnormal tissue functions.

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