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Clonal Characterization of Bone Marrow Derived Stem Cells and Their Application for Bone Regeneration

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Abstract

Tissue engineering allows the design of functionally active cells within supportive bio-scaffolds to promote the development of new tissues such as cartilage and bone for the restoration of pathologically altered tissues. However, all bone tissue engineering applications are limited by a shortage of stem cells. The adult bone marrow stroma contains a subset of nonhematopoietic cells referred to as bone marrow mesenchymal stem cells (BMSCs). BMSCs are of interest because they are easily isolated from a small aspirate of bone marrow and readily generate single-cell-derived colonies. These cells have the capacity to undergo extensive replication in an undifferentiated state *ex vivo*. In addition, BMSCs have the potential to develop either *in vitro* or *in vivo* into distinct mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma. Thus, BMSCs are an attractive cell source for tissue engineering approaches. However, BMSCs are not homogeneous and the quantity of stem cells decreases in the bone marrow in aged population. A sequential loss of lineage differentiation potential has been found in the mixed culture of bone marrow stromal cells due to a heterogenous population. Therefore, a number of studies have proposed that homogenous bone marrow stem cells can be generated from clonal culture of bone marrow cells and that BMSC clones have the greatest potential for the application of bone regeneration *in vivo*.

Keywords bone morrow, stem cell, mesenchymal, clonal culture, regenerative medicine, differentiation

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Backgound

Large bone defects, due to pathological and traumatic reasons, face great clinical challenges because of the shortage of donor bone tissue. Autologous bone graft is considered the best option, but has the limitation of donor sites. At present, tissue engineering is a new approach for bone regeneration and endeavours to repair large bone losses using 3 dimensional (3-D) scaffolds to deliver vital cells to the defective site. However, all the bone tissue engineering applications are limited by a shortage of stem cells.

Bone marrow contains a subclass of stem-like cells that are precursors of non-hematopoietic tissues. Friedenstein originally referred to them as fibroblastic colony-forming units (CFU-f) (Friedenstein *et al*., 1970). Stem cells are undifferentiated multipotent precursor cells that share two characteristic properties: unlimited or prolonged self-renewal and potency for differentiation. Therefore, stem cell-based therapies hold promise for treating degenerative disorders and injuries (Owen and Friedenstein, 1988; Cancedda *et al*., 2003; Derubeis *et al*., 2004). When stem cells head down the pathway toward differentiation, they usually proceed by first giving rise to a more specialized kind of stem cell called precursor cell or "progenitor cell", which can in turn either proliferate through self-renewal or produce fully specialized or differentiated cells.

Despite the launch of preliminary human trials and the great variety of the data available, a number of fundamental questions still need to be resolved. For example, it has been noted that a sequential loss of lineage differentiation potential occurs in the mixed culture of bone marrow stromal cells due to the heterogeneity of the cell population (Ng *et al*., 2004). One notable question that arises is whether these precursor cells are pluripotential and homogenous, that is, true stem cells, or whether they are a mixture of cells committed to various lineages of differentiation (specialized progenitors). In particular, it is not known if all, or only some, of these precursors are osteogenic. In addition, little is known about the biochemical and molecular phenotype of the starting cell populations. Although several cell surface markers can be used to recognize BMSC, no unique marker for these cells is known (Minguell *et al*., 2001). Thus, it is difficult to estimate the *in vitro* culture homogeneity, and to identify BMSC niches *in vivo*. Although some mesenchymal lineage-inducing agents are known, the molecular details regulating the lineage development still need to be investigated (Ringe *et al*., 2002a; 2002b).

Bone marrow stromal cells — Heterogenous nature

Cultured BMSCs are an interesting target for use in cell and gene therapy because of the ease with which they proliferate and give rise to differentiated progeny that can substitute for the diseased counterpart (Johnstone *et al*., 1998). Bone marrow has been defined as a complex tissue comprised of hematopoietic precursors, their differentiated progeny, and a connective tissue network referred to as stroma (Bruder *et al*., 1997a; 1997b). The marrow stromal tissue is a heterogeneous mixture of cells including adipocytes, reticulocytes, endothelial cells, and fibroblastic cells which are in direct contact with the hematopoietic elements. It has been recognised by many investigators that the adherent cell layer emerging from primary marrow cultures is composed of different cell populations (Benayahu *et al*., 1991). Our current study found that primary culture of bone marrow stromal cells contained at least three types of cells. Based on morphologic appearance they are spindle shaped cells, star shaped cells, and large flat cells. Furthermore, large numbers of genes have been found to be differently expressed between individual samples within the same group when cultured bone marrow cells were analysed by microarray study. Figure 1A shows the individual expression of 35 000 genes in three bone marrow cell samples collected from healthy juveniles while Figure 1B shows gene expression in three bone marrow cell samples from healthy adults. Over 150 genes showed a two-fold difference between samples within the juvenile group and more than 124 genes showed a two-fold difference in expression between samples within adult group. Heterogenous cell populations of bone marrow samples may therefore be the major reason for the large gene variation between individual samples. Hence, the purification of stem cell population from the mixed culture of bone marrow samples is imperative to understand the nature of mesenchymal stem cells located in the bone marrow.

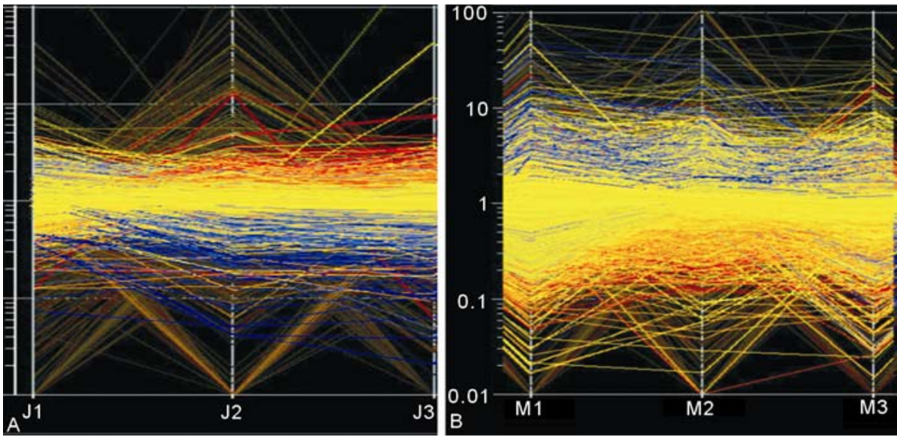


Figure 1 Microarray results from three individual bone marrow samples of juvenile patient (A) and adults (B)

Each line indicates a single gene expressed in three samples.

Clone culture and *in vitro* expansion of BMSCs

BMSCs are of interest because they are easily isolated from a small aspirate of bone marrow and readily generate single cell derived colonies. Recently, several stem-cell-related surface marker proteins have been used for the isolation and functional characterization of human mesenchymal stem cells (Bruder *et al*., 1997; Barry *et al*., 1999). However, mesenchymal stem cells express mesenchymal, muscle, epithelial, and endothelial cell surface markers (Minguell *et al*., 2001), and the absence of a unique antibody profile for mesenchymal progenitor or stem cells has hampered the purification of stem cells from a mixed population using cell surface marker selection.

The single cell-derived colonies of BMSC can be expanded through as many as 50 population doublings in about 10 weeks, and they can differentiate into osteoblasts, adipocytes, chondrocytes, myocyte, astrocytes, oligodendrocytes, and neurons (Kuznetsov *et al*., 1997). Hence, the hypothesis is that single clonal culture of bone marrow cells provides a unique approach to characterize the composition of a marrow stromal cell population. Currently, in our lab, we have successfully established and stored 19 clones from three patient's bone marrow samples (Figure 2). Single cells were isolated from the primary bone marrow culture and subsequently explanted. Of 192 clonal cell cultures from each patient (total 576 clonal cultures), only 19 clones could be continuously cultured and stored. These clones showed a continued proliferation and were able to reach confluence in 75 flasks in less than 8 weeks under conditioned culture. These results demonstrated the possibility of establishing the method of single cell culture for subsequent analysis, and it is promising in harvesting clones of stem cells from bone marrow (Xiao *et al*., 2009; Mareddy *et al*., 2007; Mareddy *et al*., 2009).

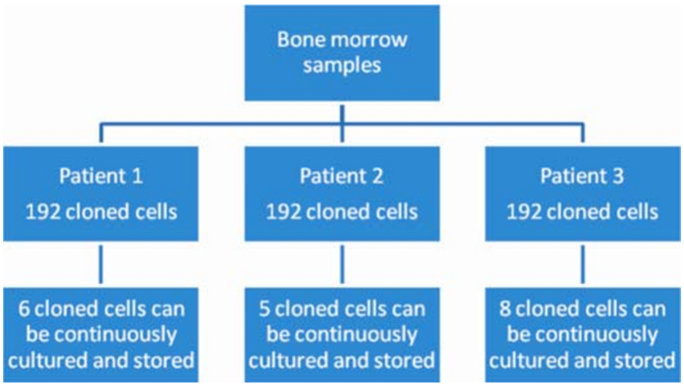


Figure 2 Clone numbers generated from the single cell culture of three BMSC samples

FGF-2 growth factor and its significance in BMSC culture

With extended culture in the conditions currently in use, human BMSCs display a tendency to lose their multipotentiality proliferation potential and *in vivo* bone forming efficiency (Banfi *et al*., 2000). Further, their use in gene and cell therapy requires their *in vitro* expansion and calls for the investigation of culture conditions required to preserve these cells as a stem cell compartment with high differentiative potential during their life span.

Basic fibroblast growth factor (FGF-2) acts as a pleiotropic factor mediating aspects of bone formation and resorption. Regulated expression and activation of basic FGF and its receptors are critical in normal skeletal development. In skeletal tissues, FGF-2 is produced by cells of the osteoblastic lineage, accumulates in bone matrix, and acts as an autocrine/paracrine factor. It has been reported that, among the many growth factors intrinsic to the skeletal tissues, FGF-2 or basic FGF is recognized as a potent mitogen for a variety of mesenchymal cells and is the most effective growth factor in promoting mesenchymal stem cell proliferation (Martin *et al*., 1997; Kawaguchi *et al*., 2001). Previous studies have shown that FGF-2 is a more potent mitogen for immature mesenchymal cells, prechondrocytes, and preosteoblasts than for differentiated osteoblasts. The addition of FGF-2 to primary cultures of BMSC helps to maintain their osteogenic potential and this effect is associated with a longer telomere size in the cultured cells (Bianchi *et al*., 2003). FGF-2 supplementation prolongs the life span of bone marrow stromal cells to more than 70 doublings and maintains their differentiation potential until 50 doublings. It has been suggested that FGF-2 *in vitro* selects for the survival of a particular subset of cells enriched in pluripotent mesenchymal precursors and is useful in obtaining a large number of cells with preserved differen- tiation potential for mesenchymal tissue repair (Bianchi *et al*., 2003).

In our clone cell culture medium, supplemented with FGF-2, a single clone cell can keep its spindle morphology and can be enriched (Figure 3), whereas in standard cell culture medium with 10% FCS, cell morphology started to change to a round shape and cell growth stopped after a few passages. It has been noted that not a single clone is able to reach the stage of cell confluence in a T75 flask without FGF-2.

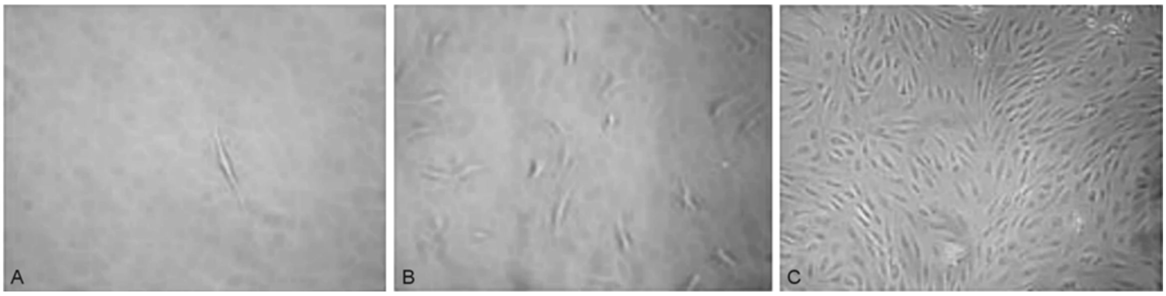


Figure 3 A single cell derived from BMSC that reached confluence after two weeks when cultured under standard medium supplemented with FGF-2

A: shows a single cell in a 96-well culture plate; B: shows cell proliferation after 3 days. C: shows cells nearly confluent after two weeks in 96-well plate.

Telomere length and telomerase activity during cell division

A number of studies (Awaya *et al*., 2001; Baerlocher *et al*., 2003; Ning *et al*., 2003; Parsch *et al*., 2004) have focussed on the effect of the *in vitro* expansion on the replicative capacity of mesenchymal stem cells by correlating their rate of telomere loss during *in vitro* expansion with their behaviour *in vivo*. Telomere shortening is the cause of replicative senescence of mammalian cells in culture and may be a cause of cellular aging *in vivo*. Telomeres are composed of the tandem DNA repeats and associated proteins that cap the ends of linear chromosomes. They provide stability to the chromosome and protect against DNA loss associated with cellular replication. Telomeres are maintained by the reverse transcriptase, telomerase. The regulation of telomere length and telomerase activity is a complex and dynamic process that is tightly linked to cell cycle regulation. There is a significant decline of telomeres length and telomerase activity in the long term culture of BMSCs (Singh *et al*., 2008; 2009). The clones generated from BMSCs require substantial passages to obtain enough cells for the telomere length and telomerase analysis. Therefore, it is likely that no telomerase activity is detectable from clone cultures.

Genes responsible for osteoblast differentiation

Verfaille *et al*. assessed the genetic pathways involved in osteoblast differentiation (Verfaillie *et al*., 2002; Verfaillie, 2002; Schwartz *et al*., 2002). Osteogenesis is a strictly controlled developmental process in which numerous extrinsic factors, including hormones and growth factors, activate osteoblast-specific signalling proteins and transcription factors (TFs) required for osteoblast differentiation. TFs Cbfa1/osf2 have been shown to regulate the expression of genes that characterize the osteoblast phenotype, including osteocalcin (OCN), osteopontin (OPN), type I collagen, bone sialoprotein (BSP), and collagenase-3. *Cbfa-1* deficient mice lack bone formation because of a maturation arrest of osteoblasts. Molecular and genetic evidence has demonstrated that *Cbfa1/osf2* activates osteoblast differentiation during embryonic development in mice and humans. In addition, it can induce osteoblast differentiation of non-osteoblastic cells.

Clonal characterization of bone marrow stromal cells

Our present studies (Xiao *et al*., 2009; Mareddy *et al*., 2007; Mareddy *et al*., 2009), based on single cell clonal cultures have demonstrated a wide variation in the proliferation rates among the 14 individual clones derived from three patients' bone marrow samples. There is even a marked variation within clones from the same sample. These wide variations in cell replication are directly related with differentiation potential. Fast growing clones have demonstrated multipotentiality, whereas slow growing clones have shown a limited differentiation potential, as well as changes in cell morphology and signs of cell senescence. The morphological changes and decreased proliferation are signs of BMSCs of a committed lineage. These findings indicate the importance to develop protocol for identifying fast growing cells from heterogeneous population in bone marrow samples for potential cell based therapy. Therefore, cell surface markers have been explored in comparing the fast and slow growing clones as well as the mixed bone marrow samples. However there is no difference in the expression of known phenotype markers currently most used for MSCs amongst fast growing clones, slow growing clones, and mixed BMSCs. All clones expressed the putative mesenchymal markers CD29, CD44, CD73, CD90, CD105, CD166, and MHC class I, but did not express the haematopoietic markers CD34, CD45, and MHC class II (Figure 4).

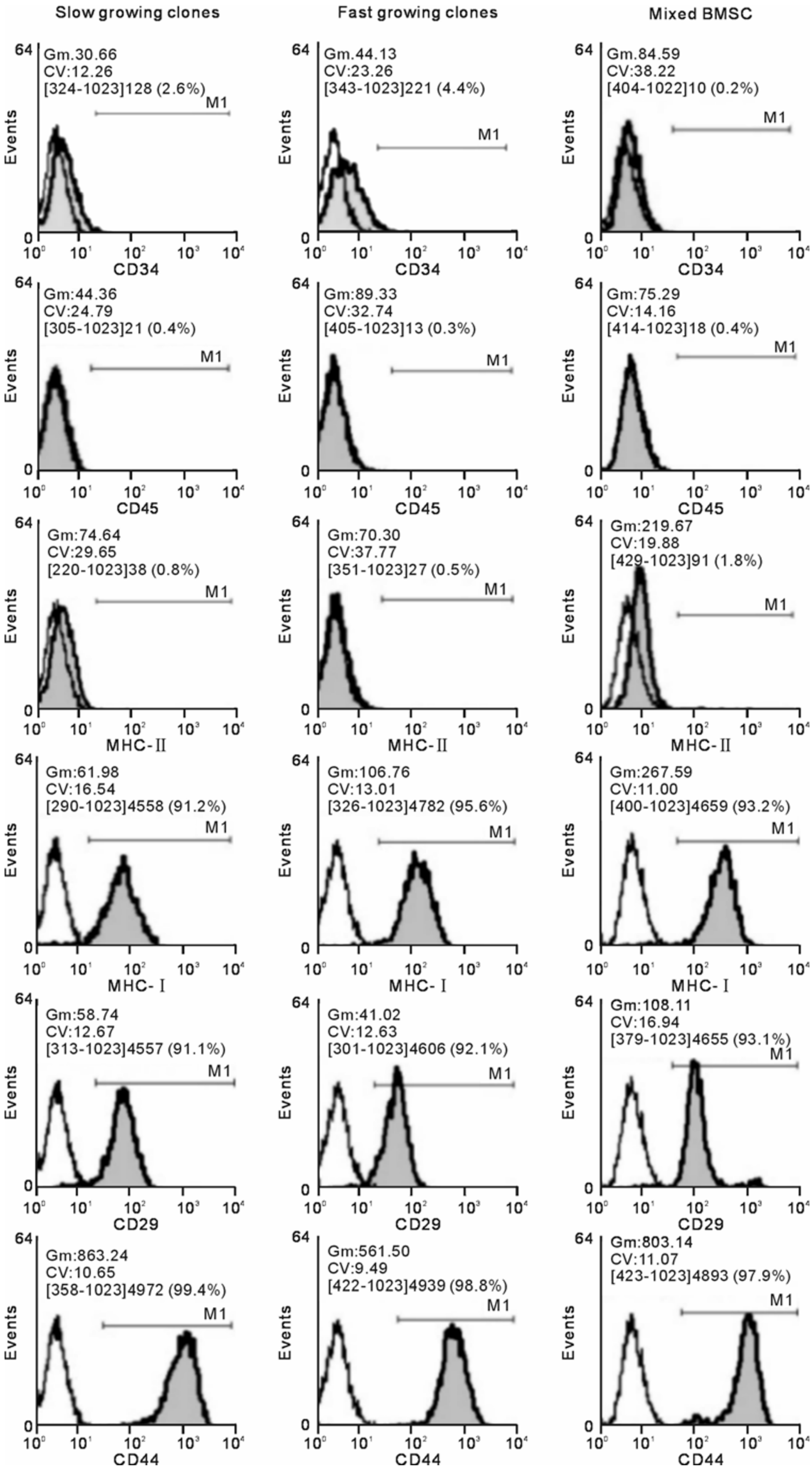
This finding indicates that there is a strong need to investigate for novel cell surface characteristic markers of BMSCs. It will be of interest to find a unique marker which can identify fast growing multipotential cells early in culture.

Molecular regulators in clone culture and stemness

A number of factors have been attributed to different stem cell properties of MSC clones such as the microenvironment MSC harboured and culture conditions (Digirolamo *et al*., 1999; Gregory *et al*., 2005). We have confirmed that slow-growing cells expressed higher level of cell senescence related genes such as p53, p16, and Rb1 compared with fast-growing cells. The specific molecular mechanisms must underlie the stemness of the clones exhibiting superior *ex vivo* expansion potential. It is revealed a pool of ten up-regulated genes which are common in all the fast-growing clones as opposed to the genes expressed in slowly-growing clones. They are associated with the maintenance of self-renewal and lineage markers of a wide array of cell types (embryonic, neural and endodermal), in addition to those specific for MSCs. They include genes encoding proteins involved in the maintenance of embryonic stem cell renewal and endodermal organogenesis such as Sox2 and Fox A2 (Wan *et al*., 2004), expression markers associated with chondrogenesis such as ACAN and COL2A1, and growth factors such as BMP2 and IGF1, which are involved in both cell proliferation, as well as induction of differentiation in a context-dependent manner (Sandell *et al*., 1999). Other genes that were expressed include NOTCH1 and DLL3, which are involved in stem cell maintenance in diverse niches (Duncan *et al*., 2005; Conboy *et al*., 2005) and cell cycle regulators, FGF2 and CDC2 (Nurse *et al*., 1993; Martin *et al*., 1997).

Immunological properties of the *in vitro* cultured BMSC and their *in vivo* bone forming efficiency

Allogeneic organ or tissue transplantation involves use of powerful immunosuppressive drugs, carrying undesirable side effects to prevent immunological rejection of the transplanted tissue. In the absence of this immunosuppression, the patient’s T-lymphocytes and natural killer cells (NK) recognize surface molecules on the transplanted cells as


Slow growing clones
Fast growing clones
Mixed BMSC
Events
64
0
M1
10⁰ 10¹ 10² 10³ 10⁴
Gm.30.66
CV:12.26
[324-1023]128 (2.6%)
Gm.44.13
CV:23.26
[343-1023]221 (4.4%)
Gm.84.59
CV:38.22
[404-1022]10 (0.2%)
CD34
Gm:44.36
CV:24.79
[305-1023]21 (0.4%)
Gm:89.33
CV:32.74
[405-1023]13 (0.3%)
Gm:75.29
CV:14.16
[414-1023]18 (0.4%)
CD45
Gm:74.64
CV:29.65
[220-1023]38 (0.8%)
Gm:70.30
CV:37.77
[351-1023]27 (0.5%)
Gm:219.67
CV:19.88
[429-1023]91 (1.8%)
MHC-Ⅱ
Gm:61.98
CV:16.54
[290-1023]4558 (91.2%)
Gm:106.76
CV:13.01
[326-1023]4782 (95.6%)
Gm:267.59
CV:11.00
[400-1023]4659 (93.2%)
MHC-Ⅰ
Gm:58.74
CV:12.67
[313-1023]4557 (91.1%)
Gm:41.02
CV:12.63
[301-1023]4606 (92.1%)
Gm:108.11
CV:16.94
[379-1023]4655 (93.1%)
CD29
Gm:863.24
CV:10.65
[358-1023]4972 (99.4%)
Gm:561.50
CV:9.49
[422-1023]4939 (98.8%)
Gm:803.14
CV:11.07
[423-1023]4893 (97.9%)
CD44

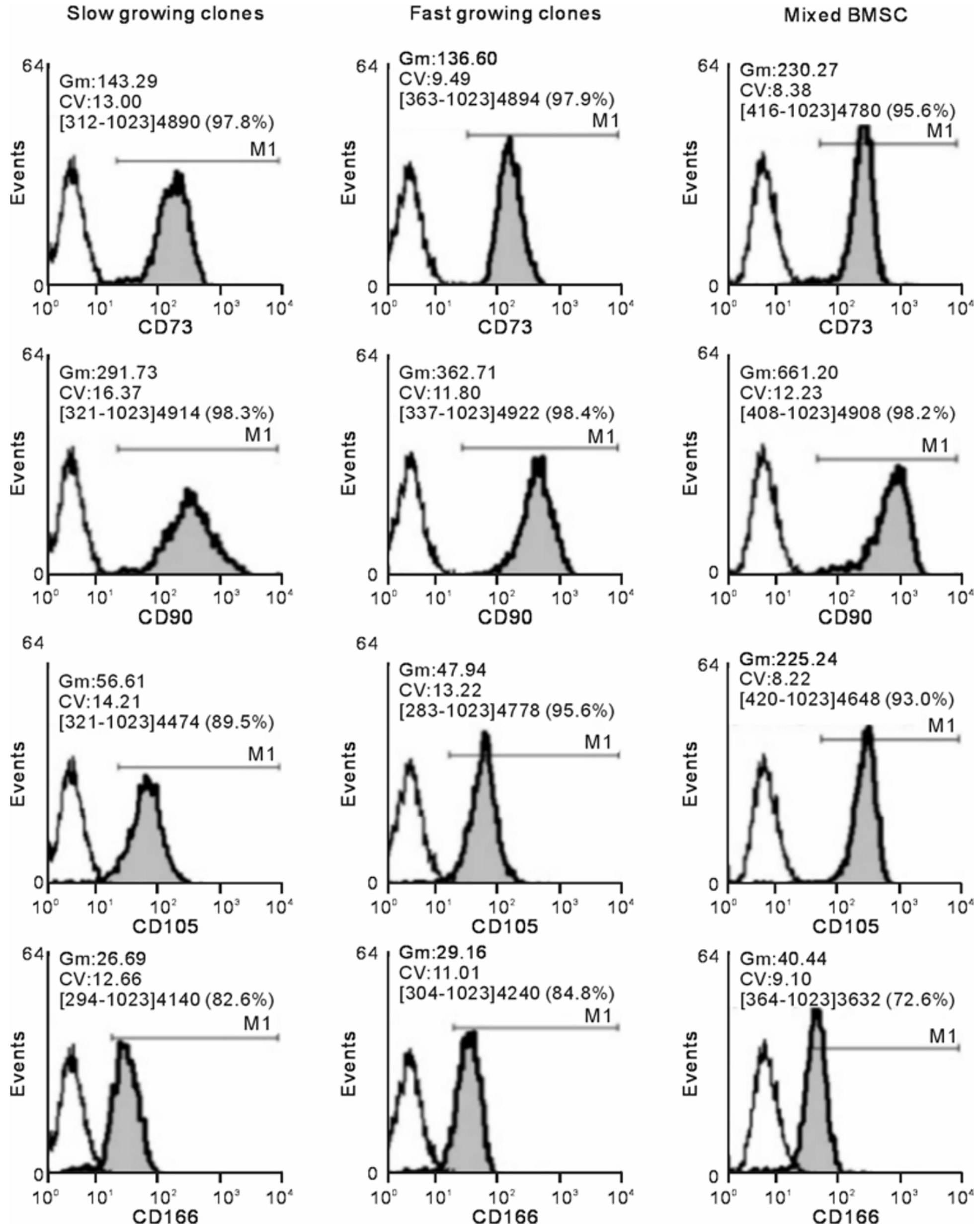


Figure 4 FACS analysis of surface epitopes in clonal cultures and mixed culture

Clonally isolated BMSC cultured for 20 PDs and the mixed cultures were labeled with PE or FITC-conjugated Abs against human CD29, CD34, CD44, CD45, CD73, CD90, CD105, CD166, MHC-I, and MHC-II or Ig isotope controls. Fluorescence intensity corresponds to the percentage of cells with specific antibody staining profile (shaded peak towards right side of the plot), as compared to nonspecific fluorescence of a cell population stained with the secondary antibody (IgG1 PE). The *x*-axis corresponds to the fluorescence intensity and the *y*–axis corresponds to the cell count. Histogram shows the flow cytometry results of the surface epitopes of the fast growing clones, slow growing clones, and the mixed BMSC population.

"foreign" and attack and destroy the cells. Also, in whole organ transplantation, donor T-lymphocytes and natural killer cells, entering the recipient from the transplanted organ, can also destroy the tissues of the transplant recipient (called "graft versus host" disease).

Interestingly, Drukker showed that embryonic stem cells (ESCs) *in vitro* express very low levels of the immunologically crucial major histocompatibility complex class I (MHC-I) proteins on their cell surface (Drukker *et al*., 2002). The presence of MHC-I proteins increases moderately when the ESCs become differentiated, whether *in vitro* or *in vivo*. A more pronounced increase in

MHC-I antigen expression has been observed when the ESCs are exposed to gamma-interferon, a protein produced in the body during immune reactions. To study the feasibility of human BMSC transplantation, the *in vitro* immunogenicity of MSCs and their ability to function as alloantigen presenting cells need to be evaluated. Early studies showed that human MSCs did not induce allogeneic T cells to proliferate, even when their major histocompatibility complex (MHC) class 2 antigen was upregulated and a co-stimulatory signal was provided by an anti-CD28 antibody (Tse and Egalka, 2002). Additionally it has been found that intravenous infusion of allogenic, major histocompatibility mismatched BMSC into baboons is well tolerated in most animals and prolonged survival of skin allografts (Bartholomew *et al*., 2001; 2002). Investigations with regard to the immune suppressive behaviour of MSCs and their immunomodulatory aspects would support the possibility that transplantation of human MSCs might be accomplished with minimal or no host suppression.

In summary, the cellular and molecular properties of BMSCs may be generated from projects designed to (1) isolate bone marrow stem cells using clone culture method; (2) identify genetic differences between stem cells and specialized progenitors and compare their immunogenicity; and, (3) test the purified bone marrow stem cells for long-term engraftment of bone formation *in vivo*.

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