J. Cell. Mol. Med. Vol 8, No 3, 2004 pp. 301-316

Stem Cell Review Series

Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy

D. Baksh ‡, L. Song ‡, R. S. Tuan*

Cartilage Biology and Orthopaedics Branch,
National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health,
Department of Health and Human Services, Bethesda, MD, USA

Received: September 14, 2004; Accepted: September 24, 2004

Abstract

A considerable amount of retrospective data is available that describes putative mesenchymal stem cells (MSCs). However, there is still very little knowledge available that documents the properties of a MSC in its native environment. Although the precise identity of MSCs remains a challenge, further understanding of their biological properties will be greatly advanced by analyzing the mechanisms that govern their self-renewal and differentiation potential. This review begins with the current state of knowledge on the biology of MSCs, specifically with respect to their existence in the adult organism and postulation of their biological niche. While MSCs are considered suitable candidates for cell-based strategies owing to their intrinsic capacity to self-renew and differentiate, there is currently little information available regarding the molecular mechanisms that govern their stem cell potential. We propose here a model for the regulation of MSC differentiation, and recent findings regarding the regulation of MSC differentiation are discussed. Current research efforts focused on elucidating the mechanisms regulating MSC differentiation should facilitate the design of optimal *in vitro* culture conditions to enhance their clinical utility cell and gene therapy.

Keywords: mesenchymal stem cells - stem cell niche - differentiation - Wnt - gene therapy

* Correspondence to: Rocky S. TUAN
Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis, and Musculoskeletal and Skin Diseases
50 South Dr., Room 1503, MSC 8022
National Institutes of Health, Bethesda, MD 20892-8022, USA.
Tel.: 301-451-6854, Fax: 301-435-8017
E-mail: tuanr@mail.nih.gov
‡These authors contributed equally.

Introduction

Mesenchymal stem cells (MSCs) have generated a great deal of excitement and promise as a potential source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiate into functional cell types that constitute the tissue in which they exist. MSCs are considered a readily accepted source of stem cells because such cells have already demonstrated efficacy in multiple types of cellular therapeutic strategies, including applications in treating children with *osteogenesis imperfecta* [1], hematopoietic recovery [2], and bone tissue regeneration strategies [3]. More importantly, these cells may be directly obtained from individual patients, thereby eliminating the complications associated with immune rejection of allogenic tissue. Despite diverse and growing information concerning MSCs and their use in cell-based strategies, the mechanisms that govern MSC self-renewal and multilineage differentiation are not well understood and remain an active area of investigation. Therefore, research efforts focused on identifying factors that regulate and control MSC cell fate decisions are crucial to promote a greater understanding of the molecular, biological and physiological characteristics of this potentially highly useful stem cell type.

Existence of mesenchymal stem cells

To date, there is no unequivocal evidence indicating that MSCs exist *in vivo*. Nevertheless, conventional wisdom promotes the existence of such a cell type, as connective tissue formation, the functional endpoint of MSC lineage development, occurs in an organism during development and throughout postnatal growth, repair and regeneration. Further support of their putative existence is derived from the important role of subpopulations of stromal cells in providing appropriate environmental cues essential for normal adult hematopoiesis [4, 5].

Due to the lack of a single definitive marker and knowledge regarding the anatomical location and distribution of MSCs *in vivo*, the demonstration of their existence has relied primarily on retrospective assays. The gold standard assay utilized to identify MSCs is the colony forming unit-fibroblast (CFU-F) assay which, at minimum, identifies adherent, spindle-shaped cells that proliferate to form colonies [6]. Some of the earliest experimental evidence supporting the existence of MSCs originated from the pioneering work of Friedenstein *et al.*, who first demonstrated that bone marrow derived-cells were capable of osteogenesis [7]. Accordingly, this assay has been used as an *in vitro* correlate for MSC potential. One of the most important caveats of this assay involves its assumption that putative MSCs can only be identified by their inherent ability to adhere, proliferate and develop on a static surface. Therefore, the primary question introduced by this system is whether these adhesion-derived cells definitively correlate to an *in vivo* population of MSCs.

Since the early work of Castro-Malaspina *et al.* [8], many researchers have employed different methods to isolate MSCs, in both serum and serum-deprived conditions, and have developed novel approaches to isolate purified populations of MSCs. These advances have furthered our understanding of MSC biology but have also created differences in terminology and read-out measures (i.e., based on morphology, phenotype, gene expression, and combinations thereof) for describing the adherent-capable cells derived from many adult tissue sources displaying fibroblast-like morphology (Table 1). Although none of these terms can accurately account for both the developmental origin and differentiation capacity of these cells, the term 'mesenchymal stem cell' (MSC) is currently most often employed. However, both this and the other named cell types depend, for their definition, on the adherence of a population of harvested cells to a tissue culture substrate, and therefore none can represent the actual progenitors existant in adult human marrow. Despite considerable amount of retrospective data available that describe the putative MSCs, the existence of a single MSC *in vivo* remains to be determined.

The mesenchymal stem cell niche

There is much research interest in determining what defines and constitutes the mesenchymal stem cell niche. It is clearly described that distinct niches exist within the bone marrow that support

Table 1 Representative examples of terms given to mesenchymal stem cells.

Term	Cell type(s) identified	Animal Source/Reference(s)
Precursors of non-hematopoietic tissue	Adherent cells of bone marrow that include fibroblast-like cells, endothelial cells, and monocytes/macrophage	Guinea pig [6] Mouse [88]
Colony forming unit-fibroblast (CFU-F)	Colonies of fibroblastic cells, with the occasional monocyte/macrophage present	Human [8] Mouse [89, 90] Rabbit [91]
Mesenchymal stem cells (MSCs)	Cells defined by their selective attachment to a solid surface	Human [92]
Marrow stromal cells	Adherent cells of bone marrow that include and/or adherent fibroblast-like cells, endothelial cells and colonies monocytes/macrophage	Mouse [39, 93, 94]
Bone marrow stromal [stem] cells [BMSSCs] and/or Stromal precursors cells (SPCs)	Non-hematopoietic cells of mesenchymal origin, displaying fibroblastic morphology	Mouse [95] Human [86, 96]
RS-1, RS-2, mMSCs (RS: Recycling stem cell) (m: mature)	RS-1: thin, spindle-shaped cells RS-2: moderately thin, spindle-shaped cells mMSCs: wider, spindle-shaped cells	Human [27, 97]
Multipotent adult progenitor cells (MAPCs)	Culture-derived bone marrow-derived progenitor cells	Humans [98] Murine [25] Rat [25]

hematopoietic stem cell (HSC) survival and growth, by providing the requisite factors and adhesive properties to maintain their viability, while facilitating an appropriate balanced output of mature progeny for the lifetime of an organism [9]. It has also been determined that these niches are formed by stromal precursor cells, specifically osteoblasts [5]. The stroma, and stromal cells, together, provide a physical support for maturing precursors of blood cells, and serve as a repository of a broad range of cell-derived cues and signals driving the commitment, differentiation and maturation of hematopoietic cells [10-12]. Specifically, endothelial cells, adipocytes, macrophages, reticular cells, fibroblasts, osteoprogenitors, HSCs and their progeny are the primary cellular components of the marrow stroma [13, 14]. It is within this dynamic and cellular microenvironment where MSCs are presumed to exist. The question, however, is: Do MSCs reside in their own unique stem niche amidst hematopoietic stem cells or do they share the same niche with hematopoietic cells? It may be argued that these two cell compartments occupy the same niche, given the close physical proximity to one another of both hematopoietic and mesenchymal cells in the bone marrow. However, the extracellular and/or intercellular signals that are required to maintain both the hematopoietic and mesenchymal stem cell developmental program in the bone marrow microenvironment are likely to be vastly different. A complete characterization of the cellular, biochemical, and molecular interactions of MSCs within their niche is needed in order to understand how these cells can be optimally regulated *in vitro*.

Despite the fact that bone marrow is considered a well-accepted source of MSCs, MSCs have been isolated from other tissue sources, including trabec-

Table 2 Examples of human MSC frequency and phenotypic properties calculated from representative studies.

Study	Cell fraction isolated	Frequency	Major cell properties
Castro-Malaspina et al., [8]	1.07 g/ml	68 – 10 in 5 x 10^6	• Adherent fibroblastic-like cells
Lazarus et al., [99]	70% Percoll (1.03 g/ml)	1 in 1 x 10^5	• Adherent fibroblastic-like cells • $CD45^-$, CD14
Pittenger et al., [19]	70% Percoll (1.073 g/ml)	1 in 1 x 10^5	• Adherent fibroblastic-like cells • $SH2^+$, $SH3^+$, $CD29^+$, $CD44^+$, $CD71^+$, $CD90^+$, $CD106^+$, $CD120a^+$, $CD124^+$
Koç et al., [2]	Percoll (1.073 g/ml) 23.4 – 5.9 ml BM	1.4 – 0.7 in 1 x $10^{5(a)}$	• Adherent fusiform fibroblastic-like cells • $SH2^+$, $SH3^+$, $SH4^+$, $CD45^-$, CD14, $CD34^-$
Kuznetsov et al., [100]	BM aspirates	34.2 – 6 in 1 x 10^5	• Adherent colonies of fibroblastic-like cells
Reyes et al, [101]	Ficoll-Paque (1.077 g/ml)	1 in 1 x 10^6	• Clusters of small adherent cells • $CD34^-$, CD44low, $CD45^-$, $CD117^-$, class I-HLA^-, class 2-HLA -DR $CD45^-$GlyA cells
Quirici et al., [102]	NGFR+ cells	1,584 in 1 x 10^6	$NGFR^+$ cells • Isolated fraction consists of small round cells that rapidly adhere to plastic • $NGFR^+$ cells express $CD34^+$ (44.1 – 45.8%), $CD113^+$ (49.4 – 29.9%) • Minority of cells expressed SH2, CD90, TE7
Gronthos et al., [47]	STRO-1+ VCAM+	1 in 3 STRO1+ VCAM+ cells	• Adherent fibroblastic-like cells (> 50 cells) with occasional cluster of cells (>10–50 cells) • 0.02% STRO-1^+ $VCAM^+$ cells in BM MNC population • >90% of cells stained for collagen type 1 • $CD45^-$ • Quiescent in vivo • No detection of mature mesenchymal cell markers (i.e. osteopontin, parathyroid hormone receptor, Cbfa1/Runx2, osterix).
Suva et al., [103]	Ficoll-Paque (1.077 g/ml)	1 in 13,000	• $CD45^-$, $CD14^-$, $CD34^-$, $CD11b^-$, $CD90^+$, HLA–ABC^+

(a) A mean of 1.4 – 0.7 x 10^5 MSCs are recovered at the first passage from 1 x 10^6 input BM MNC.

ular bone [15], adipose tissue, synovium, skeletal muscle, lung, deciduous teeth (reviewed in Tuan *et al.* [16]), and human umbilical cord perivascular cells derived from the Wharton's Jelly [17], suggesting that the MSC niche may not be restricted to just bone marrow. These findings reveal that MSCs are diversely distributed *in vivo*, and as a result may occupy a ubiquitous stem cell niche.

Key characteristics of MSCs phenotype

Considerable progress has been made towards characterizing the cell surface antigenic profile of human bone marrow-derived MSC populations using fluorescence activated cell sorting (FACS) and magnetic bead sorting techniques. To date,

however, a single marker that definitively delineates the *in vivo* MSCs has yet to be identified, due to the lack of consensus from diverse documentations of the MSC phenotype [18-21] (Table 2). However, analyses using a combination of monoclonal antibodies raised against surface markers of *in vitro*-derived MSCs (e.g., STRO-1, SH2, SH3, SH4) [18, 22] have shown some promise toward immuno-phenotyping these cells. On the other hand, the fact that MSCs share common features with endothelial, epithelial and muscle cells (reviewed in Minguell *et al.* [20]) and present a highly variable profile of cell surface antigens [23-25] makes it a daunting task to identify a universal single marker for MSCs. Despite this controversy of what defines a 'mesenchymal stem cell', there is general agreement that MSCs lack typical hematopoietic antigens, namely, CD45, CD34 and CD14 [19].

Self-renewal potential

One of the defining characteristics of stem cells is their self-renewal potential, the ability to generate identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism). The absolute self-renewal potential of MSCs remains an open question, due in large part to the different methods employed to derive populations of MSCs and the varying approaches used to evaluate their self-renewal capacity. As a population, bone marrow derived MSCs have been demonstrated to have a significant but highly variable self-renewal potential during *in vitro* serial propagation [26, 27]. Continuous labeling of fresh bone marrow cell harvests with tritiated thymidine reveals that CFU-Fs are not cycling *in vivo* [28], and their entry into cell cycle and subsequent development into colonies depend on serum growth factors [8]. In fact, higher population doublings (i.e. >50 PDs) have been achieved as a consequence of the addition of specific growth factors [e.g., fibroblast growth factor-2 (FGF-2)], to the basal culture medium [29]. Cell seeding density also plays a role in the expansion capacity of MSCs. For example, Colter *et al* [27] demonstrated that higher expansion profiles of MSCs can be attained when plated at low density (1.5-3 cells/cm^2) but not at high density (12 cells/cm^2), resulting in a dramatic increase in the fold expansion of total cells (2,000-fold vs. 60-fold expansion, respectively). This work and other similarly reported work (reviewed in Bianco *et al.* [30]) strongly suggest that MSCs and isolated MSC clones are heterogeneous with respect to their self- renewal capacity.

Multilineage differentiation potential

The multilineage differentiation potential of MSC populations derived from a variety of different species has been extensively studied *in vitro* since their first discovery in 1960s [31]. These studies demonstrate that populations of bone marrow-derived MSCs from human, canine, rabbit, rat, and mouse have the capacity to develop into terminally differentiated mesenchymal phenotypes both *in vitro* and *in vivo*, including bone [26, 32], cartilage [33], tendon [34, 35], muscle [36, 37], adipose tissue [38, 39], and hematopoietic-supporting stroma [39] (Fig. 1A). The ability of MSCs to differentiate into a variety of connective tissue cell types has rendered them an ideal candidate cell source for clinical tissue regeneration strategies, including the augmentation and local repair and regeneration of bone [33, 40], cartilage [41] and tendon [34].

Individual colonies derived from single MSC precursors have also been reported to be heterogeneous in terms of their multilineage differentiation potential. For instance, Pittenger *et al.* [19] reported that only one-third of the initial adherent bone marrow-derived MSC clones are pluripotent (osteo/chondro/adipo). Furthermore, nonimmortalized cell clones examined by Muraglia *et al.* [42] demonstrated that 30% of the *in vitro* derived MSC clones exhibited a tri-lineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage potential (osteo). These observations are consistent with other *in vitro* studies using conditionally immortalized clones [43-45]. Additionally, Kuznetsov *et al.* [46] demonstrated that only 58.8% of the single colony-derived clones had the ability to form bone within hydroxyapatite-tricalcium phosphate ceramic scaffolds after implantation in immunodeficient mice. Similar results were reported by using purer populations of MSCs maintained *in vitro* [47]. Taken together, these results suggest

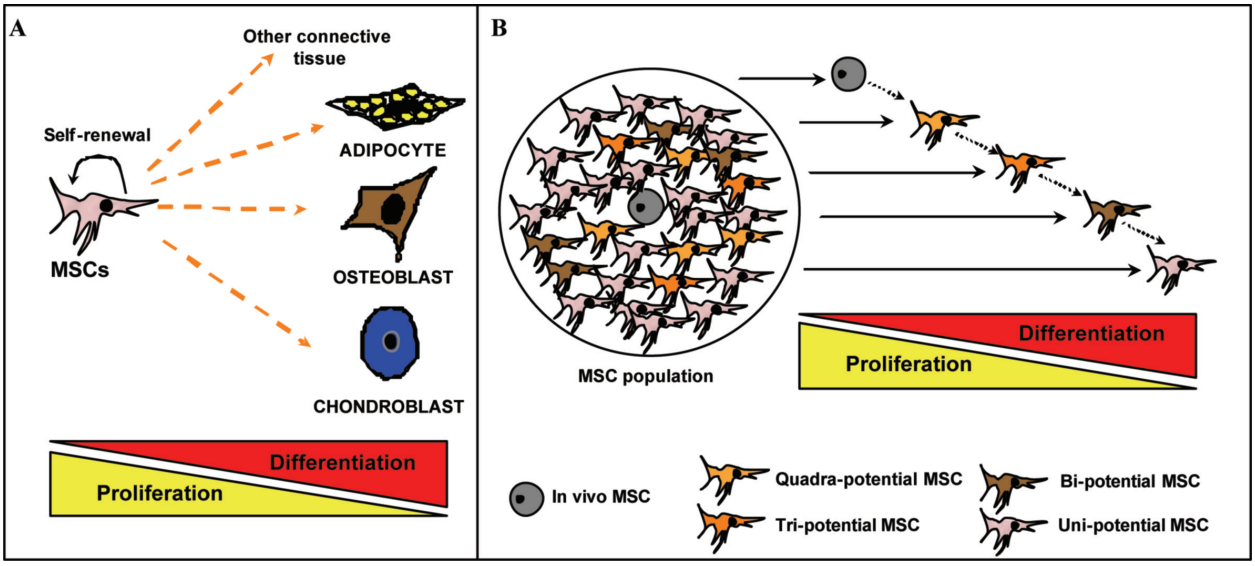

Fig. 1 Models of mesenchymal stem cell differentiation. (**A**) In this theoretical model, a mesenchymal stem cell (MSC) has the capacity to differentiate into all connective tissue cell types, including bone, cartilage, tendon, muscle, marrow, fat and dermis. Furthermore, MSCs have the potential for self-renewal and proliferation and, under defined environmental cues, can commit to a particular differentiation pathway. The lineage-committed cell progresses through several stages of maturation prior to the onset of terminal differentiation, which is marked by the cessation of proliferative capacity and shift toward synthesis of tissue-specific markers, including components of the extracellular matrix. (B) An alternative model illustrating that *in vivo*, MSCs comprise a cell population that consists of mesenchymal cells, which have different differentiation potentials (i.e., quadra-, tri-, bi and uni-potential). During in vitro culture, all or a subset of these mesenchymal cells are isolated. During differentiation, the proliferative potential of these different mesenchymal cells decreases and, depending on the initial state of differentiation, both their proliferative and multilineage potential become limited.

that clonally-derived MSCs are heterogeneous with respect to their developmental potential.

The heterogeneity of adult MSCs, demonstrated in both *in vivo* and *in vitro* studies, with respect to their self-renewal and differentiation potential, could be explained by the notion that in bone marrow, the MSC pool comprises not only putative "mesenchymal stem cells" but also subpopulations at different states of differentiation (Fig. 1B). In this model, MSCs in the bone marrow constitute a primitive stem cell population (multipotent MSCs), similar to the hematopoietic stem cell system that is capable of extensive self-renewal and formation of all the differentiated connective tissues, as well as MSCs with different multilineage potential (e.g., quadra-, tri-, bi-, and uni-potential MSCs). These various multi-potential MSCs have limited self-renewal capacity and give rise to specific cell types with terminally differentiated phenotype. The multi-potent MSCs are eventually depleted from the MSC pool during long-term culture, due to their low frequency in relation to more differentiated MSC phenotypes, present at higher frequency in the primary tissue source. The question, therefore, is how can these highly multipotent MSCs be maintained during *in vitro* culture expansion.

Several strategies have been employed to enhance and maintain the multilineage potential of MSCs, such as culturing cells with specific growth factors, enriching cells prior to initial plating, and/or culturing cells in a non-contact suspension culture configuration. However, the general approach to the culture of MSCs involves isolating the mononucleated cells containing MSCs from bone marrow aspirates and seeding these cells on tissue culture plates at a standard plating density in a minimal essential medium base containing fetal bovine serum (FBS). Within 24-48 hours, nonadherent hematopoietic cells are removed, and the adherent cells are cultured and passaged to expand the MSC population [26, 48]. Under this condition, cells can be expanded typically to 40 PDs until their

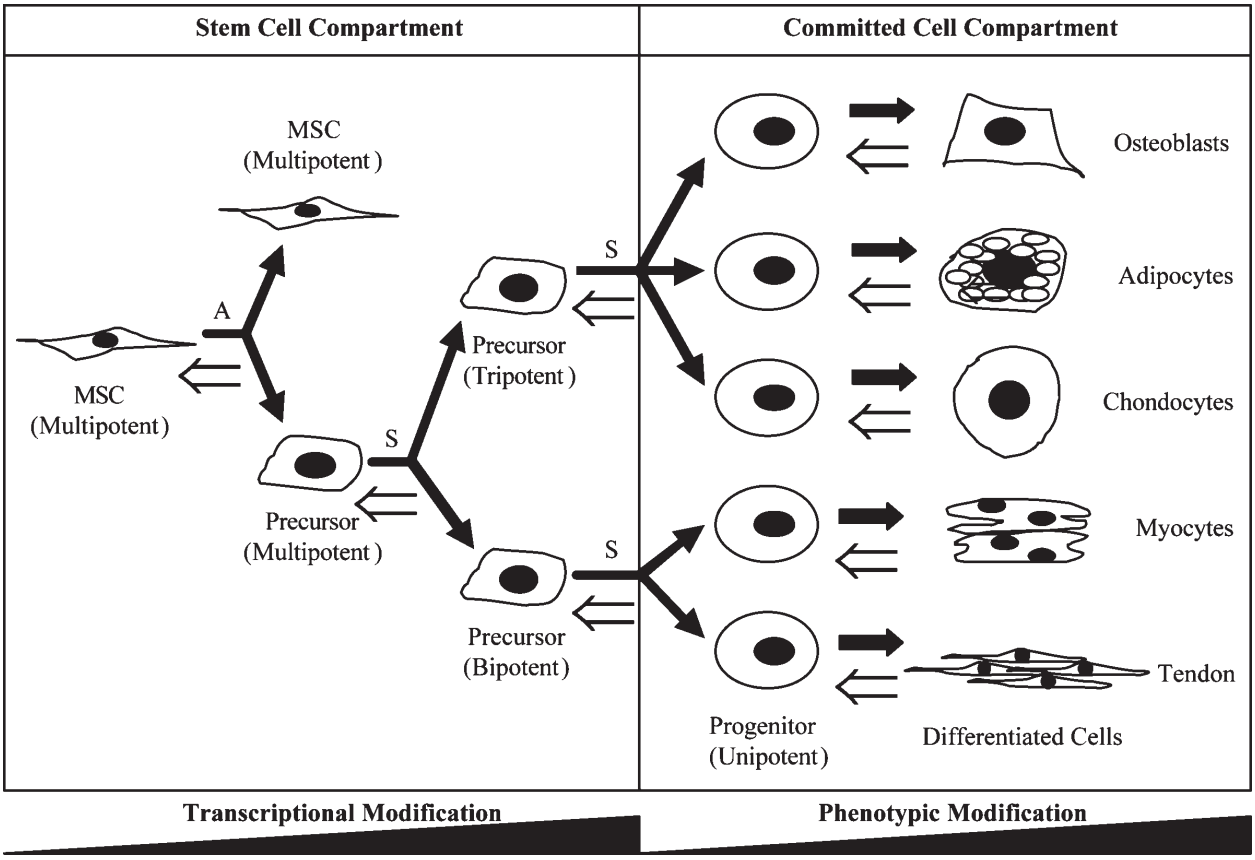

Fig. 2 Schematic model depicting adult stem cell differentiation. Uncommitted MSCs undergo two stages, occurring in the stem cell compartment and the committed cell compartment, prior to acquiring specific phenotypes. In the stem cell compartment, multipotent MSCs give rise to a less potent cell population via asymmetric cell division (A), which then generate more precursor cells with less self-renewal capacity and more restricted differentiation potential via symmetric division (S). In the committed cell compartment, these tri-or bi-potent precursor cells continue to divide symmetrically and generate bi- or unipotent progenitor cells with pre-determined cell fate, which eventually give rise to fully differentiated cells. Recent studies also suggest that the fully committed cells are able to dedifferentiate into more potent cells, and acquire a different phenotype under inductive cues (open arrows).

growth rate is significantly reduced. Furthermore, addition of specific growth factors in the MSC cultures has resulted in selective enrichment of different subsets of MSCs [25, 29, 49]. For example, supplementation of FGF-2 in the presence of 10% FBS prolongs the lifespan of bone marrow-derived MSCs to more than 70 PDs and maintains their differentiation potential until 50 PDs [29]. These results suggest that FGF-2 preferentially selects for the survival of a particular subset of MSCs with a higher self-renewal potential. Enrichment of a more homogeneous MSC starting population, particularly those that have a multilineage differentiation potential (*i.e.*, quadra- vs. bipotent cells) could also prolong the life-span of MSCs during *in vitro* expansion. A number of techniques have been developed to fulfill this purpose, such as cell size-based physical enrichment, plating property-based selection (low vs. high plating densities) [27, 50], and cell surface marker selection [22, 47, 51]. Since these approaches usually generate diverse results with respect to the expansion potential of the isolated cells, it is apparent that a clearly established, efficient, and reproducible method to the isolation, culture and expansion of putative MSCs has yet to be developed. An optimal culturing strategy would involve recapitulating the *in vivo* environment of MSCs. It has been reported that non-hematopoietic cells that display fibroblastic cell morphology, under CFU-F assay conditions, can be isolated from

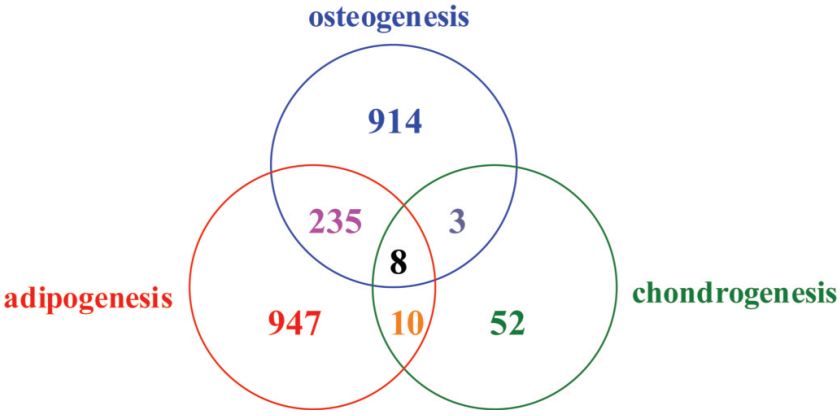

Fig. 3 Venn diagram showing the number of candidate genes that are upregulated during MSC commitment into osteoblasts (osteogenesis), adipocytes (adipogenesis), and chondrocytes (chondrogenesis), and those that are common to two or all three lineages (see text for details).

biological fluids, including adult peripheral blood and fetal blood [52, 53]. These cells show characteristics of adherent-derived MSCs in that they share a similar phenotypic profile ($CD45^-$, $CD42^+$, $SH2^+$, $SH3^+$, $SH4^+$) and have the capacity to differentiate into a variety of mesenchymal tissues (*i.e.*, bone, cartilage and adipose) both *in vitro* and *in vivo* [52-54]. These results suggest that *in vitro* derived MSCs might be able to survive and proliferate in a non-adherent environment, such as that already demonstrated in a stirred suspension culture system [55]. The suspension cells grown under these non-contact conditions maintain their ability to form functional connective tissue types. Importantly, this approach provides an alternative strategy to expand adult bone marrow-derived non-hematopoietic progenitor cell numbers in a scalable and controllable bioprocess and also provides new insight into, and possibilities to explore, mesenchymal stem/progenitor cell biology.

Regulation of differentiation

As state above, an important feature about MSCs is their multilineage differentiation potential. Under defined inductive conditions, MSCs are able to acquire characteristics of cells derived from embryonic mesoderm, such as osteoblasts, chondrocytes, adipocytes, tendon cells, as well as cells possessing ectodermal and neuronal properties. However, the molecular mechanisms that govern MSC differentiation are incompletely understood. Based on the genetic and genomic information provided by various studies, we propose a model for the regulation of adult stem cell differentiation, which incorporates two continuous yet distinct compartments (Fig. 2). In the first compartment, MSCs undergo transcriptional modification, generating precursor cells without apparent changes in phenotype and self-renewal capacity. Similar to MSCs residing in adult bone marrow, the majority of MSCs cultured *in vitro* remain quiescent and growth arrested in G0/G1, until stimulated, for example, by the supplementation of growth factors. Upon stimulation, multipotent, uncommitted MSCs undergo asymmetric division, giving rise to two daughter cells, one being the exact replica of the mother cell and maintaining multilineage potential, and the other daughter cell becoming a precursor cell, with a more restricted developmental program. In this model, the precursor cell continues to divide symmetrically, generating more tripotent and bipotent precursor cells. These tripotent and bipotent precursor cells are morphologically similar to the multipotent MSCs, but differ in their gene transcription repertoire, and therefore, still reside in the stem cell compartment. The progression of MSCs to precursor cells is considered the first step in stem cell commitment. The transition or exit from the 'stem cell compartment' to the 'commitment compartment' occurs when precursor cells continue to divide symmetrically to generate unipotent progenitor cells, simultaneous with the acquisition of lineage specific properties, rendering them fully committed mature cells with distinguishable phenotypes. At present, what is not fully understood is the mechanism that governs the transit of uncommitted stem cells to partially committed precursor or progenitor cells, and then to fully differentiated cells. To better understand this phenomenon, a number of questions need to be answered. For example, is there a common regulatory pathway that functions

as a master 'switch' that can be manipulated to turn on stem cell differentiation? How do precursor and progenitor cells selectively differentiate into one specific phenotype but not the other? Can pre-determined progenitor cells change their commitment and phenotype? Do fully differentiated cells retain multipotentiality?

The commitment and differentiation of MSCs to specific mature cell types is a tightly and temporally controlled process, involving the activities of various transcription factors, cytokines, growth factors, and extracellular matrix molecules. Global gene expression profiling using DNA microarray technology is a useful tool to identify genes involved in stem cell commitment and differentiation as a function of different inductive microenvironments. In fact, this approach has already been used successfully to identify genes that regulate osteogenic, adipogenic, and chondrogenic differentiation of MSCs [56, 57], which has greatly facilitated our effort to elucidate the mechanism controlling adult stem cell differentiation. However, although studies focused on individual lineage(s) could identify the genes essential for specific lineage (s), they often failed to identify genes that might be involved in more than one differentiation lineages, i.e., the master control genes. To determine if such master control genes exist, we have compared the transcriptome profiles associated with three mesenchymal lineages derived from human MSCs, namely, osteoblasts, chondrocytes, and adipocytes, to that of uncommitted MSCs using Affymetrix human genome U133 array set (Song and Tuan, manuscript in preparation). Genes that showed 1.5-fold or higher levels of increased expression during differentiation were selected and categorized into three subclasses, depending on their upregulation in only one lineage, in two lineages, or in all three lineages. Among 39,000 transcripts analyzed for osteogenesis, adipogenesis, and chondrogenesis, respectively, 914, 947, and 52 genes increased their expression in one mesenchymal lineage, while 235, 3, and 10 genes shared upregulated expression between two lineages (Fig. 3). Most interestingly, there are 8 genes whose expression are increased during all three mesenchymal lineage differentiation, suggesting that they might function in all three lineages, and thus may represent the putative master control genes. These genes are identified as period homolog1 (PER1), nebulette (NEBL), neuronal cell adhesion molecule (NRCAM), FK506 binding protein 5 (FKBP5), interleukin 1 type II receptor (IL1R2), zinc finger protein 145 (ZNF145), tissue inhibitor of metalloproteinase 4 (TIMP4), and serum amyloid A2. The function of these genes cover a broad range of cellular processes, including cell adhesion, protein folding, organization of actin cytoskeleton, as well as inflammatory response, implying that the initiation and commitment of adult stem cells is a complex process requiring the coordination of multiple molecules and signaling pathways. Functional analysis of these genes is necessary to determine if and how they are involved in the progression of stem cells from one differentiation stage to the next. The fact that osteoblasts and adipocytes shared more upregulated genes during their phenotypic acquisition (235 genes), compared to 3 genes shared between osteoblasts and chondrocytes, and 10 genes shared between chondrocytes and adipocytes, also implies that osteoblasts and adipocytes might share a common precursor, while chondrocytes are derived from a different precursor. Further analysis of shared genes among different lineages should advance our understanding of the hierarchical sequence of stem cell commitment during development.

The conventional view of linear hierarchical progression of stem cells from one differentiation stage to the next during their phenotypic determination (Fig. 1A) has been challenged by the recent findings that adult stem cells can give rise to cells other than their residing tissues upon *in vivo* transplantation [58-60]. Using an *in vitro* differentiation strategy, we recently showed that MSC-derived, fully differentiated osteoblasts, adipocytes, and chondrocytes can switch their phenotypes to other mesenchymal lineages in response to specific extracellular stimuli [61]. During the transdifferentiation process, extensive cell proliferation is observed and committed cells lose their lineage-specific phenotype before resuming a cell state similar to primitive stem cells, both in morphology and function. Furthermore, upon induction, these dedifferentiated cells are able to acquire a new differentiated phenotype, that is, undergo redifferentiation (Fig. 4). Taken together, it is reasonable to conclude that both pre-committed progenitor cells and fully differentiated cells retain the multipotentiality, and that their plasticity during 'phenotypic switching' can be preserved during differentiation and be

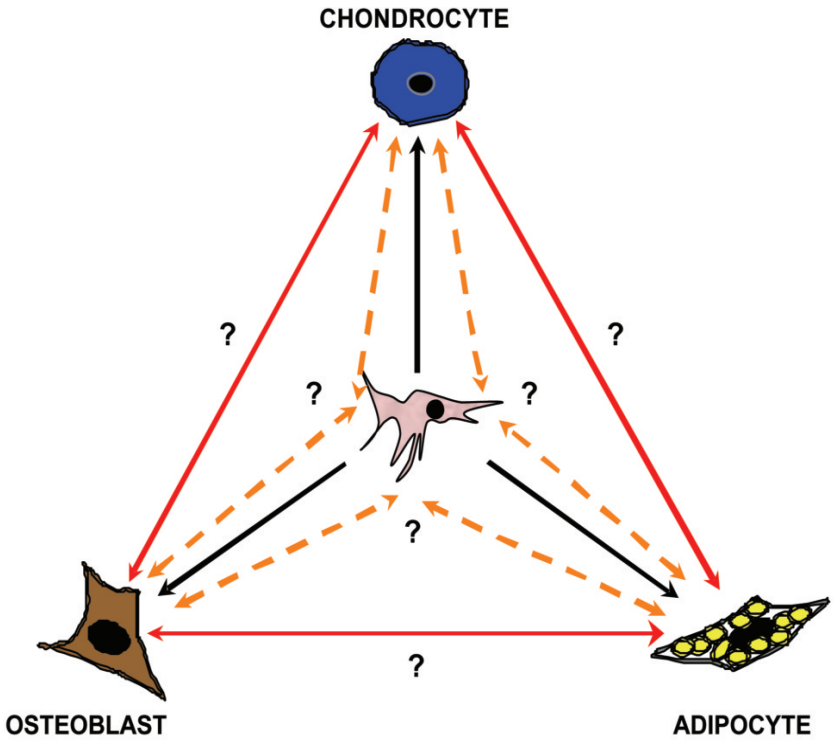

Fig. 4 Model of mesenchymal stem cell plasticity. Experimental evidence has demonstrated the ability of MSCs to transdifferentiate and dedifferentiate as a function of specific culture conditions. MSCs have the potential to differentiate into osteoblasts, chondrocytes and adipocytes (solid black arrows), and may also transdifferentiate directly into other mature connective tissue cell types (solid red arrows). However, these differentiated cells from MSCs are also able to re-enter a proliferation stage and resume the characteristics of undifferentiated MSCs through genomic reprogramming (dashed orange lines). At this stage, these cells can become a new connective tissue cell type. Factors or signals involved in maintaining the MSC biological properties (question marks) require further investigation.

reaquired under defined, appropriate microenvironmental circumstances, such as tissue repair and regeneration.

Studies using transgenic and knockout mice and human musculoskeletal disorders have provided valuable information on how MSC differentiate into multiple lineages during embryonic development and adult homeostasis [62]. On the other hand, analyses of *in vitro* differentiation of MSCs under appropriate conditions that recapitulate the *in vivo* process have led to the identification of various factors essential for stem cell commitment. Among them, secreted molecules and their receptors (*e.g.*, transforming growth factor-β), extracellular matrix molecules (*e.g.*, collagens and proteoglycans), actin cytoskeleton, and intracellular transcription factors (e.g., Cbfa1/Runx2, PPARγ, Sox9, and MEF2) play important roles in driving the commitment of multipotent stem cells into specific lineages, and maintain their differentiated phenotypes [63-66]. For example, osteogenesis of MSCs, both *in vitro* and *in vivo*, is a well-orchestrated sequence of events, involving multiple steps and expression of various regulatory factors. During osteogenesis, multipotent MSCs undergo asymmetric division and generate osteoprecursors, which then progress to form osteoprogenitors, preosteoblasts, functional osteoblasts, and eventually osteocytes [61]. This progression from one differentiation stage to the next is accompanied by the activation and subsequent inactivation of transcription factors, *i.e.*, Cbfa1/Runx2, Msx2, Dlx5, Osx, and expression of bone-related marker genes, i.e., osteopontin, collagen type I, alkaline phosphatase, bone sialoprotein, and osteocalcin [66, 67]. Disruption of the timely sequential expression of these genes results in the delay of the cell's progression to the osteoblast phenotype and the subsequent failure to form functional osteoblasts.

Members of the Wnt family have recently shown to impact MSC osteogenesis [68, 69]. Wnts are a family of secreted cysteine-rich glycoproteins that have been implicated in the regulation of stem cell maintenance, proliferation, and differentiation during embryonic development. Canonical Wnt signaling increases the stability of cytoplasmic β-catenin by receptor-mediated inactivation of GSK-3 kinase activity and promotes β-catenin translocation into the nucleus. The active β-catenin/TCF/LEF complex then regulates the transcription of genes involved in cell proliferation

and differentiation. In humans, mutations in the Wnt co-receptor, LRP5, lead to defective bone formation. Gain of function mutation results in high bone mass, whereas loss of function causes an overall loss of bone mass and strength, indicating that Wnt signaling is positively involved in embryonic osteogenesis. Canonical Wnt signaling pathway also functions as a stem cell mitogen, via the stabilization of intracellular β-catenin and activation of the β-catenin/TCF/LEF transcription complex, resulting in activated expression of cell cycle regulatory genes, such as Myc, cyclin D1, and Msx1 [70]. When MSCs are exposed to Wnt3a, a prototypic canonical Wnt signal, under standard growth medium conditions, they show markedly increased cell proliferation and a decrease in apoptosis [69], consistent with the mitogenic role of Wnts in hematopoietic stem cells [71]. However, exposure of MSCs to Wnt3a conditioned medium or overexpression of ectopic Wnt3a during osteogenic differentiation inhibits osteogenesis in vitro through β-catenin mediated down-regulation of TCF activity [69]. The expression of several osteoblast specific genes, e.g., alkaline phosphatase, bone sialoprotein, and osteocalcin, is dramatically reduced, while the expression of Cbfa1/Runx2, an early osteo-inductive transcription factor was not altered, implying that Wnt3a-mediated canonical signaling pathway is necessary, but not sufficient, to completely block MSC osteogenesis. These results raise the question of whether there are other signaling pathways involved in triggering osteogenic commitment. On the other hand, Wnt5a, a typical non-canonical Wnt member, has been shown to promote osteogenesis *in vitro* [69]. Since Wnt3a promotes MSC proliferation during early osteogenesis, it is very likely that canonical Wnt signaling functions in the initiation of early osteogenic commitment by increasing the number of osteoprecursors in the stem cell compartment, while non-canonical Wnt drives the progression of osteoprecursors to mature functional osteoblasts. Interestingly, several osteoblast marker genes, e.g., alkaline phosphatase, osteocalcin, appear to contain putative TCF/LEF binding sites. It will be of interest to determine whether the inhibitory effect of Wnt3a on osteogenesis is the direct result of suppression of osteogenic gene expression, or the secondary effect of increasing cell proliferation.

Application of MSCs in cell and gene therapy

Adult MSCs have shown great promise in cell and gene therapy applications, because of their multipotentiality and capacity for extensive self-renewal. In a large number of animal transplantation studies, MSCs expanded *ex vivo* were able to differentiate into cells of the residing tissue, repair the damaged tissue due to trauma or disease, and partially restore its normal function. They not only regenerate tissues of mesenchymal lineages, such as intervertebral disc cartilage [72], bone [73, 74], cardiomyocytes [75], and articular cartilage at knee joints [76], but also differentiate into cells derived from other embryonic layers, including neurons [77] and epithelia in skin, lung, liver, intestine, kidney, and spleen [78-80]. These applications demonstrate the plasticity of these adult stem cells and their usefulness in multiple tissue repair and regeneration and in cell therapy applications. It is also noteworthy that neither autologous nor allogeneic MSCs induce any immunoreactivity in the host upon local transplantation or systemic administration [74, 75, 79, 81], thus rendering MSCs an ideal carrier to deliver genes into the tissues of interest for gene therapy applications.

Several approaches have been examined and used to introduce exogenous DNA into MSCs to render them useful in tissue regeneration therapies. Viral transduction, particularly using adenovirus-mediated gene transfer, can generate stable cell clones with high efficiency and low cell mortality, thus making it a popular option in gene therapy. For example, MSCs infected with an adenovirus vector containing dominant-negative mutant collagen type I gene have been used successfully to repair the bone in individuals with the brittle bone disorder, osteogenesis imperfecta [73]. However, the safety concerns associated with viral transduction have prompted us to look for alternative non-viral gene delivery approaches. Traditional transfection methods, such as calcium phosphate precipitation, lipofection, and electroporation, have shown little success in delivering plasmid DNA into primary MSCs, usually resulting in less than 1% transfection efficiency and high cell mortality [82]; therefore, these methods are not suitable for producing sufficient amount of transfected cells for gene delivery and transplantation. Recently, two new methods have

been developed to transfect primary MSCs, namely Nucleofection™ and vibration-based transfection using Symphonizer™. Nucleofection™ (Amaxa Biosystems), combining electroporation and a proprietary transfection solution, has been shown to successfully introduce a GFP reporter plasmid into primary MSCs with up to 80% transfection efficiency and 50% cell viability [83]. Approximately 10% of the transfected cells retain GFP expression after 3 weeks, suggesting that the plasmid is transiently incorporated into the cell nucleus. There was no apparent adverse effects on normal cellular function as transfected cells were able to differentiate into chondrocytes at similar efficiency as untransfected cells upon induction. Song and Tuan [61] have recently demonstrated that MSCs transfected using Nucleofection™ with a lineage-specific promoter reporter, i.e., an osteocalcin promoter driven GFP plasmid, acquired osteoblast phenotype as a function of induction time and maintained their multilineage transdifferentiation capacity. Taken together, these results strongly suggest the utility of this method in delivering functional genes into MSCs used for transplantation to either promote repair and regeneration of diseased or damaged tissue or rescue defective genes.

Another recently developed method of nonvirally transfecting cells is based on electric field-induced molecular vibration using a newly introduced machine, Gene Symphonizer™ (Mollennium Inc., Japan). This non-invasive method can introduce foreign DNA into both established cell lines, such as murine C3H10T1/2 cells, and primary cells, including chondrocytes, embryonic mesenchymal cells, and MSCs, at high transfection efficiency (20-80%) with low cell mortality [82]. This approach also does not interfere with the normal cellular differentiation activities of human and chick mesenchymal progenitors. Another unique and important feature about this method is its ability to also deliver exogenous DNA into multilayered tissue, such as sternum cartilage and skeletal muscle. As such, this method could be applied to deliver foreign DNA directly into target tissue/organs *in vivo*, an ideal option for gene therapy.

Despite their enormous potential, one of the major bottlenecks in the use of MSCs has been their limited numbers, given that a variety of clinical applications require significant cell numbers to achieve a clinically successful result (*e.g.*, bone marrow transplantations and regeneration of large segmental bone defects). The yield of MSCs from the primary tissue source is insufficient for such clinical applications. Unlike embryonic stem cells, adult MSCs, which lack telomerase activity [84], show defined *ex vivo* proliferation capability, reaching senescence and losing multilineage differentiation potential after 34-50 population doublings in culture. Thus, it is necessary and critical to develop new strategies to prolong the replicative capacity of MSCs without impairing their multipotentiality. Several studies have shown that forced ectopic expression of human telomerase reverse transcriptase (hTERT) in postnatal MSCs could extend their life span to more than 260 population doublings, while maintaining their osteogenic, chondrogenic, adipogenic, neurogenic, and stromal differentiation potential [85, 86] . Importantly, these hTERT-transduced, immortalized MSCs have normal karyotype and do not cause tumor formation in xenogenic transplants, thus making them an attractive candidate source of cells for tissue repair and regeneration. However, caution must be exercised in using these immortalized MSCs since they express higher levels of osteogenic lineage specific genes, such as Cbfa1/Runx2, osterix, and osteocalcin, compared to non-transduced MSCs [87], which could potentially compromise their ability to commit to other cell lineages.

Conclusions

A growing body of research evidence has definitively demonstrated that MSCs exist in the adult tissue/organs. Despite the lack of knowledge of the origin of the putative MSCs, they have been successfully isolated from various tissue sources, mostly prominently, from bone marrow. These cells have already shown great regenerative potential. However, to continue to take advantage of these cells for cell and gene therapy applications will require a complete understanding of how the maintenance and differentiation of MSCs are regulated both *in vivo* and *in vitro*. Knowledge gained in these areas will facilitate the design of optimal *in vitro* conditions that incorporate regimes targeted towards generating highly functional MSCs for cell-based clinical applications.

References

1. **Horwitz E.M., Gordon P.L., Koo W.K., Marx J.C., Neel M.D., McNall R.Y., Muul L., Hofmann T.,** Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone, *Proc. Natl. Acad. Sci. USA*, **99:** 8932-8937, 2002
2. **Koc O.N., Gerson S.L., Cooper B.W., Dyhouse S.M., Haynesworth S.E., Caplan A.I., Lazarus H.M.,** Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy, *J Clin. Oncol.*, **18:** 307-316, 2000
3. **Petite H., Viateau V., Bensaid W., Meunier A., de Pollak C., Bourguignon M., Oudina K., Sedel L., Guillemin G.,** Tissue-engineered bone regeneration, *Nat. Biotechnol.*, **18:** 959-963, 2000
4. **Dexter T.M., Wright E.G., Krizsa F., Lajtha L.G.,** Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures, *Biomedicine.*, **27:** 344-349, 1977
5. **Calvi L.M., Adams G.B., Weibrecht K.W., Weber J.M., Olson D.P., Knight M.C., Martin R.P., Schipani E., Divieti P., Bringhurst F.R., Milner L.A., Kronenberg H.M., Scadden D.T.,** Osteoblastic cells regulate the haematopoietic stem cell niche, *Nature*, **425:** 841-846, 2003
6. **Friedenstein A.J., Chailakhjan R.K., Lalykina K.S.,** The development of fibroblast colonies in monolayer cultures of guinea- pig bone marrow and spleen cells, *Cell Tissue Kinet.*, **3:** 393-403, 1970
7. **Friedenstein A.J.,** Osteogenetic activity of transplanted transitional epithe lium, *Acta Anat. (Basel)*, **45:** 31-59, 1961
8. **Castro-Malaspina H., Gay R.E., Resnick G., Kapoor N., Meyers P., Chiarieri D., McKenzie S., Broxmeyer H.E., Moore M.A.,** Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny, *Blood*, **56:** 289-301, 1980
9. **Janowska-Wieczorek A., Majka M., Ratajczak J., Ratajczak M.Z.,** Autocrine/paracrine mechanisms in human hematopoiesis, *Stem Cells*, **19:** 99-107, 2001
10. **Koller M.R., Manchel I., Palsson B.O.,** Importance of parenchymal:stromal cell ratio for the ex vivo reconstitution of human hematopoiesis, *Stem Cells*, **15:** 305-313, 1997
11. **Strobel E.S., Gay R.E., Greenberg P.L.,** Characterization of the in vitro stromal microenvironment of human bone marrow, *Int. J. Cell Cloning*, **4:** 341-356, 1986
12. **Tavassoli M. ,Takahashi K.,** Morphological studies on long-term culture of marrow cells: characterization of the adherent stromal cells and their interactions in maintaining the proliferation of hemopoietic stem cells, *Am. J. Anat.*, **164:** 91-111, 1982
13. **Castro-Malaspina H., Ebell W., Wang S.,** Human bone marrow fibroblast colony-forming units (CFU-F), *Prog. Clin. Biol. Res.*, **154:** 209-236, 1984
14. **Wang Q.R., Wolf N.S.,** Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution, *Exp. Hematol.*, **18:** 355-359, 1990
15. **Noth U., Osyczka A.M., Tuli R., Hickok N.J., Danielson K.G., Tuan R.S.,** Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells, *J. Orthop. Res.*, **20:** 1060-1069, 2002
16. **Tuan R.S., Boland G., Tuli R.,** Adult mesenchymal stem cells and cell-based tissue engineering, *Arthritis Res. Ther.*, **5:** 32-45, 2003
17. **Sarugaser R., Lickorish D., Baksh D., Hosseini M.M., Davies J.E.,** Human umbilical cord perivascular (HUCPV) cells: A source of mesenchymal progenitors, *Stem Cells*, *In Review*, 2004
18. **Gronthos S., Graves S.E., Ohta S., Simmons P.J.,** The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors, *Blood*, **84:** 4164-4173, 1994
19. **Pittenger M.F., Mackay A.M., Beck S.C., Jaiswal R.K., Douglas R., Mosca J.D., Moorman M.A., Simonetti D.W., Craig S., Marshak D.R.,** Multilineage potential of adult human mesenchymal stem cells, *Science*, **284:** 143-147, 1999
20. **Minguell J.J., Erices A., Conget P.,** Mesenchymal stem cells, *Exp Biol. Med (Maywood.)*, **226:** 507-520, 2001
21. **Tocci A., Forte L.,** Mesenchymal stem cell: use and perspectives, *Hematol. J*, **4:** 92-96, 2003
22. **Haynesworth S.E., Baber M.A., Caplan A.I.,** Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies, *Bone*, **13:** 69-80, 1992
23. **Vogel W., Grunebach F., Messam C.A., Kanz L., Brugger W., Buhring H.J.,** Heterogeneity among human bone ma rrow-derived mesenchymal stem cells and neural progenitor cells, *Haematologica*, **88:** 126-133, 2003
24. **Simmons P.J., Torok-Storb B.,** CD34 expression by stromal precursors in normal human adult bone marrow, *Blood*, **78:** 2848-2853, 1991
25. **Jiang Y., Jahagirdar B.N., Reinhardt R.L., Schwartz R.E., Keene C.D., Ortiz-Gonzalez X.R., Reyes M., Lenvik T., Lund T., Blackstad M., Du J., Aldrich S., Lisberg A., Low W.C., Largaespada D.A., Verfaillie C.M.,** Pluripotency of mesenchymal stem cells derived from adult marrow, *Nature*, **418**:41-49, 2002
26. **Bruder S.P., Jaiswal N., Haynesworth S.E.,** Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation, *J. Cell Biochem.*, **64:** 278-294, 1997
27. **Colter D.C., Class R., DiGirolamo C.M., Prockop D.J.,** Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow, *Proc. Natl. Acad. Sci. U. S. A*, **97:** 3213-3218, 2000
28. **Friedenstein A.J., Chailakhyan R.K., Latsinik N.V., Panasyuk A.F., Keiliss-Borok I.V.,** Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo, *Transplantation*, **17:** 331-340, 1974
29. **Bianchi G., Banfi A., Mastrogiacomo M., Notaro R., Luzzatto L., Cancedda R., Quarto R.,** *Ex vivo* enrich-

ment of mesenchymal cell progenitors by fibroblast growth factor 2, *Exp. Cell Res.*, **287:** 98-105, 2003
30. **Bianco P., Riminucci M., Gronthos S., Robey P.G.,** Bone marrow stromal stem cells: nature, biology, and potential applications, *Stem Cells*, **19:** 180-192, 2001
31. **Friedenstein A.J., Piatetzky-Shapiro I.I., Petrakova K.V.,** Osteogenesis in transplants of bone marrow cells, *J Embryol. Exp Morphol.*, **16:** 381-390, 1966
32. **Bruder S.P., Kurth A.A., Shea M., Hayes W.C., Jaiswal N., Kadiyala S.,** Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells, *J Orthop. Res*, **16:** 155-162, 1998
33. **Kadiyala S., Young R.G., Thiede M.A., Bruder S.P.,** Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential *in vivo* and *in vitro*, *Cell Transplant.*, **6:** 125-134, 1997
34. **Young R.G., Butler D.L., Weber W., Caplan A.I., Gordon S.L., Fink D.J.,** Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair, *J. Orthop. Res.*, **16:** 406-413, 1998
35. **Awad H.A., Butler D.L., Boivin G.P., Smith F.N., Malaviya P., Huibregtse B., Caplan A.I.,** Autologous mesenchymal stem cell-mediated repair of tendon, *Tissue Eng*, **5:** 267-277, 1999
36. **Ferrari G., Cusella-De Angelis G., Coletta M., Paolucci E., Stornaiuolo A., Cossu G., Mavilio F.,** Muscle regeneration by bone marrow-derived myogenic progenitors, *Science*, **279:** 1528-1530, 1998
37. **Galmiche M.C., Koteliansky V.E., Briere J., Herve P., Charbord P.,** Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway, *Blood*, **82:** 66-76, 1993
38. **Dennis J.E., Merriam A., Awadallah A., Yoo J.U., Johnstone B., Caplan A.I.,** A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse, *J. Bone Miner. Res.*, **14:** 700-709, 1999
39. **Prockop D.J.,** Marrow stromal cells as stem cells for non-hematopoietic tissues, *Science*, **276:** 71-74, 1997
40. **Richards M., Huibregtse B.A., Caplan A.I., Goulet J.A., Goldstein S.A.,** Marrow-derived progenitor cell injections enhance new bone formation during distraction, *J Orthop. Res*, **17:** 900-908, 1999
41. **Johnstone B., Yoo J.U.,** Autologous mesenchymal progenitor cells in articular cartilage repair, *Clin. Orthop.*, **367**: S156-S162, 1999
42. **Muraglia A., Cancedda R., Quarto R.,** Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model, *J. Cell Sci.*, **113:** 1161-1166, 2000
43. **Majumdar M.K., Thiede M.A., Mosca J.D., Moorman M., Gerson S.L.,** Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells, *J. Cell Physiol*, **176:** 57-66, 1998
44. **Dormady S.P., Bashayan O., Dougherty R., Zhang X.M., Basch R.S.,** Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment, *J. Hematother. Stem Cell Res.*, **10:** 125-140, 2001
45. **Osyczka A.M., Noth U., O'Connor J., Caterson E.J., Yoon K., Danielson K.G., Tuan R.S.,** Multilineage differentiation of adult human bone marrow progenitor cells transduced with human papilloma virus type 16 E6/E7 genes, *Calcif. Tissue Int.*, **71:** 447-458, 2002
46. **Kuznetsov S.A., Krebsbach P.H., Satomura K., Kerr J., Riminucci M., Benayahu D., Robey P.G.,** Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation *in vivo*, *J. Bone Miner. Res.*, **12:** 1335-1347, 1997
47. **Gronthos S., Zannettino A.C., Hay S.J., Shi S., Graves S.E., Kortesidis A., Simmons P.J.,** Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow, *J. Cell Sci.*, **116:** 1827-1835, 2003
48. **Ohgushi H., Caplan A.I.,** Stem cell technology and bioceramics: from cell to gene engineering, *J. Biomed. Mater. Res.*, **48:** 913-927, 1999
49. **Gronthos S., Simmons P.J.,** The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro, *Blood*, **85:** 929-940, 1995
50. **Sekiya I., Larson B.L., Smith J.R., Pochampally R., Cui J.G., Prockop D.J.,** Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality, *Stem Cells*, **20:** 530-541, 2002
51. **Simmons P.J., Torok-Storb B.,** Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1, *Blood*, **78:** 55-62, 1991
52. **Zvaifler N.J., Marinova-Mutafchieva L., Adams G., Edwards C.J., Moss J., Burger J.A., Maini R.N.,** Mesenchymal precursor cells in the blood of normal individuals, *Arthritis Res*, **2:** 477-488, 2000
53. **Campagnoli C., Roberts I.A., Kumar S., Bennett P.R., Bellantuono I., Fisk N.M.,** Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow, *Blood*, **98:** 2396-2402, 2001
54. **Erices A., Conget P., Minguell J.J.,** Mesenchymal progenitor cells in human umbilical cord blood, *Br. J. Haematol.*, **109:** 235-242, 2000
55. **Baksh D., Davies J.E., Zandstra P.W.,** Adult human bone marrow-derived mesenchymal progenitor cells are capable of adhesion-independent survival and expansion, *Exp. Hematol.*, **31:** 723-732, 2003
56. **Doi M., Nagano A., Nakamura Y.,** Molecular cloning and characterization of a novel gene, EMILIN-5, and its possible involvement in skeletal development, *Biochem. Biophys. Res. Commun.*, **313:** 888-893, 2004
57. **Qi H., Aguiar D.J., Williams S.M., La Pean A., Pan W., Verfaillie C.M.,** Identification of genes responsible for osteoblast differentia tion from human mesodermal progenitor cells, *Proc. Natl. Acad. Sci. USA*, **100:** 3305-3310, 2003
58. **LaBarge M.A., Blau H.M.,** Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury, *Cell*, **111:** 589-601, 2002
59. **Zhao L.R., Duan W.M., Reyes M., Keene C.D., Verfaillie C.M., Low W.C.,** Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats, *Exp. Neurol.*, **174:** 11-20, 2002

60. **Jiang Y., Vaessen B., Lenvik T., Blackstad M., Reyes M., Verfaillie C.M.,** Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain, *Exp Hematol.*, **30:** 896-904, 2002
61. **Song L., Tuan R.S.,** Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow, *FASEB J.*, **18:** 980-982, 2004
62. **Roelen B.A., Dijke P.,** Controlling mesenchymal stem cell differentiation by TGFbeta family members, *J. Orthop. Sci.*, **8:** 740-748, 2003
63. **Olsen B.R., Reginato A.M., Wang W.,** Bone development, *Annu. Rev. Cell Dev. Biol.*, **16:** 191-220, 2000
64. **Waddington R.J., Roberts H.C., Sugars R.V., Schonherr E.,** Differential roles for small leucine-rich proteoglycans in bone formation, *Eur. Cell Mater.*, **6:** 12-21, 2003
65. **McBeath R., Pirone D.M., Nelson C.M., Bhadriraju K., Chen C.S.,** Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment, *Dev. Cell*, **6:** 483-495, 2004
66. **Harada S., Rodan G.A.,** Control of osteoblast function and regulation of bone mass, *Nature*, **423:** 349-355, 2003
67. **Madras N., Gibbs A.L., Zhou Y., Zandstra P.W., Aubin J.E.,** Modeling stem cell development by retrospective analysis of gene expression profiles in single progenitor-derived colonies, *Stem Cells*, **20:** 230-240, 2002
68. **De Boer J., Wang H.J., Van Blitterswijk C.,** Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells, *Tissue Eng.*, **10:** 393-401, 2004
69. **Boland G.M., Perkins G., Hall D., Tuan R.S.,** Wnt 3a promotes proliferation and supressess osteogenic differentiation of adult human mesenchymal stem cells, *J. Cell. Biochem*, *In press*, 2004
70. **Willert K., Brown J.D., Danenberg E., Duncan A.W., Weissman I.L., Reya T., Yates J.R., III, Nusse R.,** Wnt proteins are lipid-modified and can act as stem cell growth factors, *Nature*, **423:** 448-452, 2003
71. **Reya T., Duncan A.W., Ailles L., Domen J., Scherer D.C., Willert K., Hintz L., Nusse R., Weissman I.L.,** A role for Wnt signalling in self-renewal of haematopoietic stem cells, *Nature*, **423:** 409-414, 2003
72. **Crevensten G., Walsh A.J., Ananthakrishnan D., Page P., Wahba G.M., Lotz J.C., Berven S.,** Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs, *Ann. Biomed. Eng*, **32:** 430-434,
73. **Chamberlain J.R., Schwarze U., Wang P.R., Hirata R.K., Hankenson K.D., Pace J.M., Underwood R.A., Song K.M., Sussman M., Byers P.H., Russell D.W.,** Gene targeting in stem cells from individuals with osteogenesis imperfecta, *Science*, **303:** 1198-1201, 2004
74. **Arinzeh T.L., Peter S.J., Archambault M.P., van den B.C., Gordon S., Kraus K., Smith A., Kadiyala S.,** Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defe ct, *J. Bone Joint Surg. Am.*, **85-A:** 1927-1935, 2003
75. **Grinnemo K.H., Mansson A., Dellgren G., Klingberg D., Wardell E., Drvota V., Tammik C., Holgersson J., Ringden O., Sylven C., Le Blanc K.,** Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium, *J. Thorac. Cardiovasc. Surg.*, **127:** 1293-1300, 2004
76. **Barry F.P.,** Mesenchymal stem cell therapy in joint disease, *Novartis Found. Symp.*, **249:** 86-96, 2003
77. **Sugaya K.,** Potential use of stem cells in neuroreplacement therapies for neurodegenerative diseases, *Int. Rev. Cytol.*, **228:** 1-30, 2003
78. **Chapel A., Bertho J.M., Bensidhoum M., Fouillard L., Young R.G., Frick J., Demarquay C., Cuvelier F., Mathieu E., Trompier F., Dudoignon N., Germain C., Mazurier C., Aigueperse J., Borneman J., Gorin N.C., Gourmelon P., Thierry D.,** Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome, *J. Gene Med.*, **5:** 1028-1038, 2003
79. **Deng Y., Guo X., Yuan Q., Li S.,** Efficiency of adenoviral vector mediated CTLA4Ig gene delivery into mesenchymal stem cells, *Chin Med. J. (Engl.)*, **116:** 1649-1654, 2003
80. **Ortiz L.A., Gambelli F., McBride C., Gaupp D., Baddoo M., Kaminski N., Phinney D.G.,** Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects, *Proc. Natl. Acad. Sci. USA*, **100:** 8407-8411, 2003
81. **Liechty K.W., Mackenzie T.C., Shaaban A.F., Radu A., Moseley A.M., Deans R., Marshak D.R., Flake A.W.,** Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after *in utero* transplantation in sheep, *Nat. Med.*, **6:** 1282-1286, 2000
82. **Song L., Chau L., Sakamoto Y., Nakashima J., Koide M., Tua n R.S.,** Electric field-induced molecular vibration for noninvasive, high-efficiency DNA transfection, *Mol. Ther.*, **9:** 607-616, 2004
83. **Haleem-Smith H., Derfoul A., Okafor C., Tuli R., Olse D., Hall D.J., Tuan R.S.,** Optimization of high efficiency transfecrtion of adult human mesenchymal stem cells, *Molecular Biotechnology*, *Accepted*, 2004
84. **Zimmermann S., Voss M., Kaiser S., Kapp U., Waller C.F., Martens U.M.,** Lack of telomerase activity in human mesenchymal stem cells, *Leukemia*, **17:** 1146-1149, 2003
85. **Simonsen J.L., Rosada C., Serakinci N., Justesen J., Stenderup K., Rattan S.I., Jensen T.G., Kassem M.,** Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells, *Nat. Biotechnol.*, **20:** 592-596, 2002
86. **Shi S., Gronthos S., Chen S., Reddi A., Counter C.M., Robey P.G., Wang C.Y.,** Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression, *Nat. Biotechnol.*, **20:** 587-591, 2002
87. **Gronthos S., Chen S., Wang C.Y., Robey P.G., Shi S.,** Telomerase accelerates osteogenesis of bone marrow stromal stem cells by upregulation of CBFA1, osterix, and osteocalcin, *J Bone Miner. Res*, **18:** 716-722, 2003
88. **Friedenstein A.J., Gorskaja J.F., Kulagina N.N.,** Fibroblast precursors in normal and irradiated mouse hematopoietic organs, *Exp. Hematol.*, **4:** 267-274, 1976
89. **Friedenstein A.J., Latzinik N.W., Grosheva A.G., Gorskaya U.F.,** Marrow microenvironment transfer by

heterotopic transplantation of freshly isolated and cultured cells in porous sponges, *Exp. Hematol.*, **10:** 217-227, 1982
90. **Mori M., Sadahira Y., Awai M.,** Characteristics of bone marrow fibroblastic colonies (CFU-F) formed in collagen gel, *Exp. Hematol.*, **15:** 1115-1120, 1987
91. **Owen M.E., Cave J., Joyner C.J.,** Clonal analysis *in vitro* of osteogenic differentiation of marrow CFU-F, *J. Cell Sci.*, **87:** 731-738, 1987
92. **Caplan A.I.,** Mesenchymal stem cells, *J Orthop. Res*, **9:** 641-650, 1991
93. **Mori K.J., Fujitake H., Ohkubo H., Ito Y., Dexter T.M.,** Development of stromal cell colonies in bone marrow cell culture, *Gann*, **69:** 689-693, 1978
94. **Piersma A.H., Brockbank K.G., Ploemacher R.E., van Vliet E., Brakel-van Peer K.M., Visser P.J.,** Characterization of fibroblastic stromal cells from murine bone marrow, *Exp. Hematol.*, **13:** 237-243, 1985
95. **Dexter T.M., Lajtha L.G.,** Proliferation of haemopoietic stem cells in vitro, *Br. J. Haematol.*, **28:** 525-530, 1974
96. **Bianco P., Robey P.G.,** Marrow stromal stem cells, *J. Clin. Invest.*, **105:** 1663-1668, 2000
97. **Colter D.C., Sekiya I., Prockop D.J.,** Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells, *Proc. Natl. Acad. Sci. USA*, **98:** 7841-7845, 2001
98. **Reyes M., Lund T., Lenvik T., Aguiar D., Koodie L., Verfaillie C.M.,** Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells, *Blood*, **98:** 2615-2625, 2001
99. **Lazarus H.M., Haynesworth S.E., Gerson S.L., Rosenthal N.S., Caplan A.I.,** Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use, *Bone Marrow Transplant.*, **16:** 557-564, 1995
100. **Kuznetsov S.A., Mankani M.H., Gronthos S., Satomura K., Bianco P., Robey P.G.,** Circulating skeletal stem cells, *J. Cell Biol.*, **153:** 1133-1140, 2001
101. **Reyes M., Verfaillie C.M.,** Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells, *Ann. N. Y. Acad. Sci.*, **938:** 231-233, 2001
102. **Quirici N., Soligo D., Bossolasco P., Servida F., Lumini C., Deliliers G.L.,** Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies, *Exp Hematol.*, **30:** 783-791, 2002
103. **Suva D., Garavaglia G., Menetrey J., Chapuis B., Hoffmeyer P., Bernheim L., Kindler V.,** Non-hematopoietic human bone marrow contains long-lasting, pluripotential mesenchymal stem cells, *J. Cell Physiol*, **198:** 110-118, 2004