5
Bone Marrow Stromal Stem Cells for Repairing the Skeleton

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Introduction

Bone regeneration is required for the repair of skeletal defects including, but not limited to, birth defect, trauma, extirpative surgery, non-unions and segmental defects, disease processes which lead in turn to dysfunctional bone and/or low bone mass (Phillips *et al.*, 1992; Guise and Mundy, 1998). Autologous bone grafts have been used to repair a 'critical size' of bone defect (that is to say, bone defects larger than those that would heal spontaneously). The osteogenic mechanisms of autologous bone grafts are associated with a series of responses, including the formation of a haematoma around the implanted fragment of bone, local inflammation, graft necrosis, graft resorption, and eventually new bone formation. It is likely that the number of surviving osteogenic cells that have been derived from donor bone grafts is very limited. Therefore, the graft's advantage to the recipient lies in its osteoconductive and osteoinductive properties (Friedlaender, 1987). Disadvantages of autologous bone grafts are the limitation of graft quantity and the morbidity of the donor site.

Allografts, usually represented by demineralized bone matrix, have also been used to induce new bone formation (Mellonig and Bowers, 1990). Although demineralized bone allografts may lead to success in the repair of bone defects, the concern over the transmission of blood-borne diseases has limited the use of allograft bone over the last decade. Alloplastic bone substitutes, or at least those which include hydroxyapatite ceramics, titanium mesh, and methylmethacrylate paste, are used for conductive bone formation (Byrd *et al.*, 1993; Johnson *et al.*, 1996;

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Abbreviations: BMPs, bone morphogenetic proteins; BMSSCs, bone marrow stromal stem cells; BMSSCs-T, telomerase-expressing BMSSCs.

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Kresbach *et al.*, 1998). In principle, these osteoconductive materials may provide scaffolds for osteogenic cell adhesion, and may lead to the new bone formation. However, the clinical application of alloplastic bone substitutes is largely limited by their unpredictable bone formation (Verburg *et al.*, 1988). Clearly, three clinically available techniques, including autologous bone graft, allografts, and the use of alloplastic material for repairing bone defects, have their limitations; thus, development of improved methods for bone regeneration is required.

Bone marrow stromal stem cells (BMSSCs) and bone regeneration

Even though bone grafts have been used for over a century to provide new bone formation, researchers and clinicians are still continuing to pursue more efficient and practical ways to generate bone *in vivo*. The clinical application of post-natal stem cells has been demonstrated successfully in certain circumstances, such as the recovery of haematopoietic bone marrow in cancer patients using mobilized peripheral blood stem cells (Buckner, 1999; Bacigalupo *et al.*, 2000). This long-term success of clinical stem cell therapy has fuelled investigations into the potential of other stem cell populations. For tissue engineering, stem cells hold great promise for treating damaged tissue. Using stem cells with osteogenic potential to repair bone defects thus seems a reasonable approach to improve new bone formation.

Recently, it has been demonstrated that bone marrow stromal stem cells (BMSSCs) are truly post-natal stem cells, capable of differentiating into a variety of cell types, such as osteoblasts, chondrocytes, adipocytes, muscle cells, and neural cells (Prockop, 1997; Azizi *et al.*, 1998; Bianco *et al.*, 2001). They were initially identified by their capacity to form clonogenic cell clusters (CFU-F: colony-forming units-fibroblast) *in vitro*, a common feature amongst different stem cell populations (Friedenstein *et al.*, 1974; Castro-Malaspina *et al.*, 1980; Gronthos *et al.*, 2000; Uchida *et al.*, 2000; Weissman, 2000; Kuznetsov *et al.*, 2001). Each colony was seen to represent a strain of cells from a single precursor cell. Previous studies have documented that individual BMSSC colonies demonstrate marked differences in their proliferation rates *in vitro* and developmental potentials *in vivo* (Friedenstein *et al.*, 1974; Kuznetsov *et al.*, 1997; Pittenger *et al.*, 1999), indicating their important characteristic of heterogeneity. Importantly, only a proportion of single-colony derived strains (58.8%) was found to be able to form bone upon *in vivo* transplantation (Kutznetsov *et al.*, 1997).

BMSSCs are consistently able to differentiate into osteoblasts both *in vitro* and *in vivo*, without any special treatment or induction. When cultured with a particular medium, BMSSCs show the ability to form mineralized nodules, thereby demonstrating their osteogenic capability *in vitro* (Shi *et al.*, 2002; Gronthos *et al.*, 2003a). Upon *in vivo* transplantation, BMSSCs differentiate into functional osteoblasts; this system has thus far been proven to be one of the most reliable *in vivo* systems for studying the differentiation of BMSSCs and understanding the mechanisms of bone formation *in vivo* (Kresbach *et al.*, 1997; Kuznetsov *et al.*, 1997; Shi *et al.*, 2002; Gronthos *et al.*, 2003b). One of the system's most striking characteristics is that BMSSCs can generate a bone/marrow organ structure (Kresbach *et al.*, 1997; Kuznetsov *et al.*, 1997). Although the detailed mechanisms involved in the initiation and maintenance of the bone/marrow organ have yet to be determined, recent studies shed light on the dynamic process of new bone formation in immunocompromised mice.

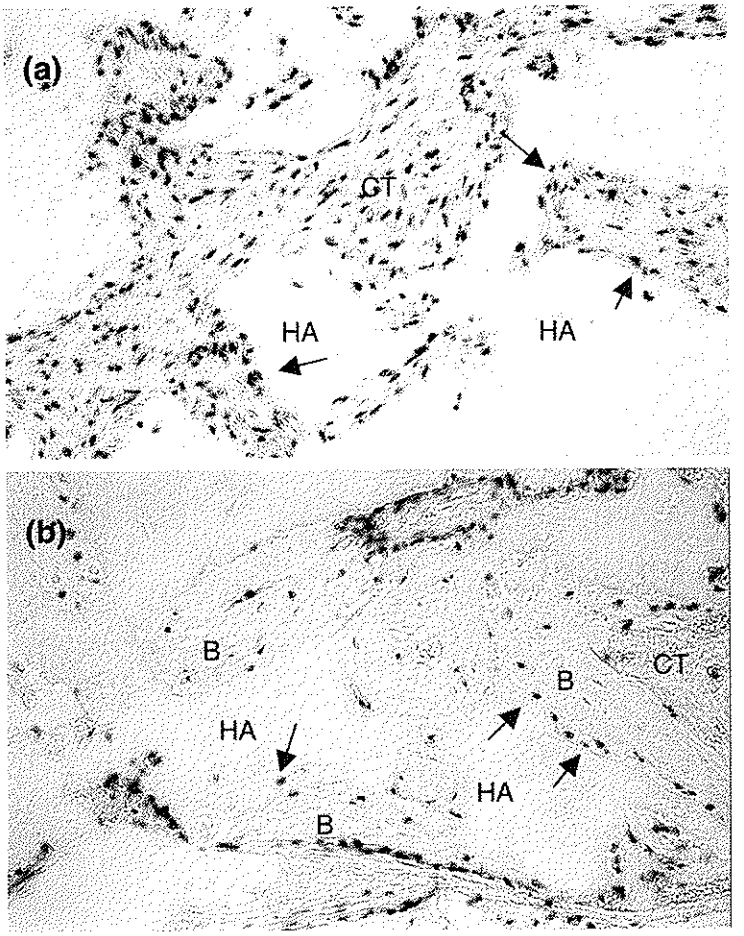

Figure 5.1. (a) The number of transplanted BMSSC is decreased after *in vivo* transplantation. Human alu *in situ* hybridization demonstrated that many transplanted BMSSC (black staining on the nuclei of cells) reside in the connective tissue compartment (*CT*) at one week post-transplantation. (b) Eight weeks after transplantation, bone (*B*) was generated on the surfaces of HA/TCP carriers (*HA*) and human cells (black staining on nuclei of cells), either differentiated into bone-forming cells (arrows) or residing in the connective tissue compartment (*CT*). The number of transplanted human BMSSC was dramatically decreased eight weeks after the transplantation. Original magnification: 40×.

Following *in vivo* transplantation, BMSSCs either attached to the surface of the HA/TCP carrier or were distributed in the connective tissue compartment during the first two weeks (*Figure 5.1*). Bone formation has been shown to start at 2–4 weeks post-transplantation, and eventually leads to the regeneration of a bone/marrow organ structure in BMSSC transplants (*Figure 5.2*). Osteogenesis is associated with a decreased number of human BMSSCs as the connective tissue compartment is replaced by recipient haematopoietic marrow elements. By 16 weeks post-transplantation, the transplants show even further formation of mineralized tissue when compared to 8-week transplants, indicating that BMSSCs have the potential to

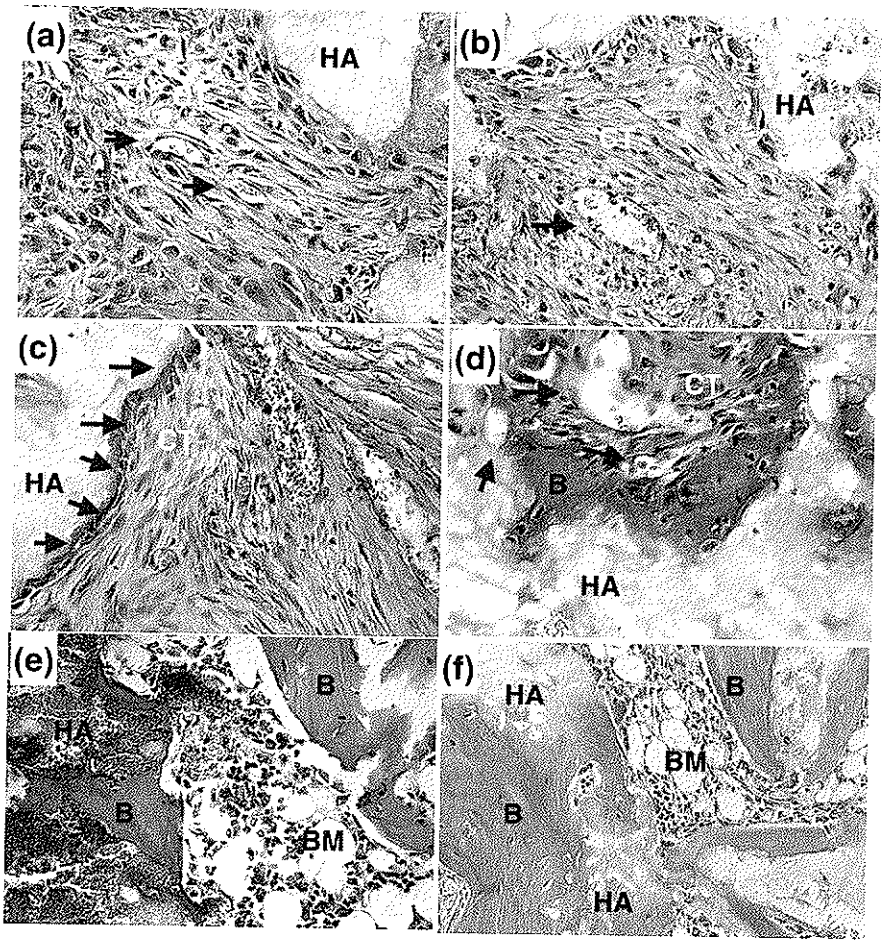

Figure 5.2. Histology of BMSSC transplants. Cross-section of a representative BMSSC transplant after 1 week (a), 2 weeks (b), 4 weeks (c), 6 weeks (d), 8 weeks (e), and 16 weeks (f) post-transplantation, stained with H&E. In the first 2 weeks post-transplantation, BMSSC transplants contain connective tissue (*CT*) around the HA/TCP carrier (*HA*), without any sign of bone formation (a and b). Ingrowth of blood vessels (arrows in a, b, and d) was found in the connective tissue compartment (*CT*). Initiation of bone formation was found on the surfaces of the HA/TCP carrier (*HA*) at 4 weeks post-transplantation of BMSSC (arrows in c). Continuation of bone formation (*B*) was conducted on the surface of HA/TCP (d) and led to bone/haematopoeitic marrow (*BM*) organ-like structure formation (e and f). Original magnification: 40×.

continue forming mineralized tissue after the organ-like structures are formed (*Figure 5.2*). This also suggests that BMSSCs are not only able to differentiate into osteoblasts *in vivo* in the early stages of transplantation, but are also capable of inducing host cells to participate in tissue regeneration by the formation of a haematopoietic marrow (*Figure 5.2*). The connective tissue compartment aids osteogenesis by supporting blood vessel in-growth and subsequent haematopoietic marrow formation in BMSSC transplants.

Pre-clinical and clinical applications of BMSSCs

In recent years, using various models including mice, rats, dog, sheep, and humans, researchers have attempted to determine whether BMSSCs are capable of forming bone for therapeutic purposes. When a tube-shaped ceramic carrier loaded with *ex vivo* expanded human BMSSCs was used to treat segmental defects in athymic mice (Bruder *et al.*, 1998a), the BMSSC/ceramics complex seemed to increase bone formation and bone strength when compared with a cell-free ceramic control group, from 4–12 weeks after operation. A similar result was also reported for dogs by the same group of researchers (Bruder *et al.*, 1998b). However, these studies lacked convincing histological evidence to reinforce their conclusions. However, other studies in which cultured mouse BMSSCs were used to repair cranial defects in immunocompromised mice provided convincing histological evidence to support a notion that BMSSCs are capable of repairing critical size cranial defects as early as 2 weeks after transplantation (Kresbach *et al.*, 1998). In addition, a number of preclinical studies in animal models showed convincing results of bone regeneration, in support of the potential clinical application of BMSSCs (Kadiyala *et al.*, 1997; Kon *et al.*, 2000; Petite *et al.*, 2000; Shang *et al.*, 2001). Recently, human osteoprogenitors isolated from patients' bone marrow were implanted at the site of overcritical sized bone defects, using macroporous hydroxyapatite scaffolds as carrier vehicles. In all three patients examined, the treatment resulted in substantial improvement in the repair of large defects in long bone (Quarto *et al.*, 2001), thus providing a promising trial in support of further studies on humans, although more evidence is required from patients to properly evaluate the therapeutic value of BMSSCs.

Genetic manipulation of BMSSCs for improving new bone formation

The evidence accumulated so far suggests that BMSSC-mediated bone formation is a more complicated process than expected. Bone-forming cells have to interact with their microenvironment to initiate osteogenesis and then maintain the regenerated bone/marrow organ structure. What an optimal microenvironment for bone regeneration is, remains to be determined. This may be one of the challenges we currently face for delivering a consistent osteogenic outcome *in vivo*. Another critical obstacle is that BMSSCs in post-natal status are designed principally for a remodelling process, in which only a small amount of bone formation may be required for maintaining the integrity of bone. However, when BMSSCs are used for bone regeneration, the repair of relatively large defects is required. Therefore, it is necessary to improve the currently available technologies for BMSSCs-mediated formation of new bone.

GENETIC MANIPULATION STRATEGIES

Genetic manipulation may help multi-potential BMSSC populations to develop cell-based therapies for the treatment of bone defects. BMP-transfected BMSSCs have been used successfully to treat large segmental femoral and calvarial defects in animal models (Lieberman *et al.*, 1998; Gysin *et al.*, 2002) by continuously

delivering osteoinductive proteins, but the mechanisms still need to be elucidated further. In addition, BMPs contributed to multiple biological processes rather than only the bone formation. The effects of BMPs on these other biological processes are as yet unknown.

Telomerase, a cellular ribonucleoprotein reverse transcriptase, is responsible for the elongation of the telomere and the prevention of the gradual loss of sequence from the ends of chromosomes so as to avoid replicative senescence *in vitro* (Nakamura *et al.*, 1997; Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). Most adult human somatic cells seem to lack telomerase activity, whereas highly proliferative germline cells, haematopoeitic stem cells, and many cancer cells appear to retain detectable levels of telomerase activity (Counter *et al.*, 1994a,b, 1995; Kim *et al.*, 1994; Broccoli *et al.*, 1995; Wright *et al.*, 1996; Yasumoto *et al.*, 1996; Shay and Bacchetti, 1997; McEachern *et al.*, 2000). Recently, several groups have reported that human adult BMSSCs and osteoblasts propagated *in vitro* contain no detectable levels of telomerase activity (Yudoh *et al.*, 2001; Shi *et al.*, 2002; Simonsen *et al.*, 2002). However, following forced ectopic expression of telomerase, the lifespan of BMSSCs was significantly increased and their proliferative capabilities were extended *in vitro*, coupled with an enhanced capacity for bone formation *in vivo* (*Figure 5.3*) (Shi *et al.*, 2002; Simonsen *et al.*, 2002). Telomerase activity appeared to maintain a larger pool of osteoprogenitors, as measured by a significant increase in the population of cells expressing the pre-osteogenic marker, STRO-1 (Shi *et al.*, 2002). In accordance with regular BMSSCs, telomerase-expressing BMSSCs (BMSSCs-T) can generate bone/marrow elements, indicating that BMSSCs-T are capable of interacting with the host microenvironment to maintain tissue homeostasis. Telomerase is not an oncogene product, such that its presence permits cell proliferation but does not cause uncontrolled proliferation or immortalization (Urquidi *et al.*, 2000). It has been identified that BMSSCs-T undergo normal apoptosis, either induced through the death-receptor pathway (CD95) or via the mitochondrial pathway, and, more importantly, they show no sign of malignant transformation *in vivo* (Shi *et al.*, 2002). Moreover, it has been found that BMSSCs-T have an accelerated and enhanced ability to form mineral under osteogenic inductive conditions *in vitro* (Gronthos *et al.*, 2003a). Similarly, xenogeneic transplantation of BMSSCs-T have yielded ectopic bone formation at 2 weeks post-transplantation, 2–4 weeks earlier than typically seen with control BMSSCs. In addition, harvested BMSSCs-T transplants were seen to contain higher numbers of human cells at 8 weeks post-transplantation when compared to the control BMSSCs (BMSSCs-C) transplants, coupled with a significant increase in new bone formation (Gronthos *et al.*, 2003a). One possible mechanism leading to accelerated osteogenesis by BMSSCs-T may be attributed, at least in part, to the up-regulation of important osteogenic genes such as those for Cbfa1, osteorix, and osteocalcin *in vitro*. Cbfa1 is a transcription factor that has been described as an important master regulatory molecule controlling early osteogenesis. This molecule acts through the activation or regulation of several downstream bone-associated genes, such as those for osterix, alkaline phosphatase, and osteocalcin, to promote later stages of osteogenic differentiation and subsequent matrix mineralization (Lian *et al.*, 1989; Ducy *et al.*, 1997; Nakashima *et al.*, 2002). However, the mechanisms by which telomerase promotes the expression of *Cbfa1*, and thus other

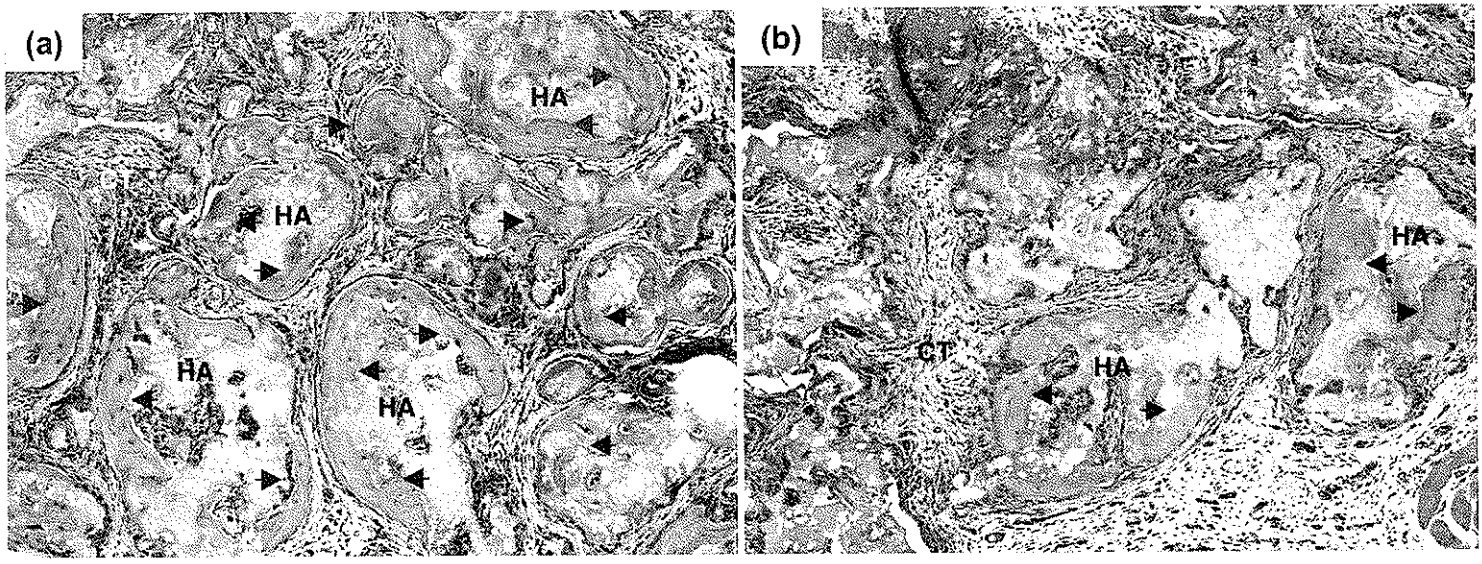

Figure 5.3. Histology of telomerase transfected BMSSC transplants. Cross-section of a representative telomerase transfected BMSSC transplant (a) and regular BMSSC (b) 8 weeks after transplantation, stained with H&E. Transplanted BMSSCs were able to generate bone (arrows) on the surface of HA/TCP carrier, which was surrounded by connective tissue (*CT*) and haematopoeitic marrow element (*HA*). Clearly, significantly more bone (arrows) was formed in telomerase transfected BMSSC transplant (a) when compared to regular BMSSC transplant (b). Original magnification: 5×.

genes, such as those for osterix and osteocalcin, in BMSSCs, requires further investigation. Nevertheless, it has been hypothesized that an increase in STRO-1 positive osteogenic precursor cells, as well as the up-regulation of Cbfa1, osterix, and osteocalcin, may be responsible for the accelerated and enhanced osteogenic capacity of BMSSCs-T (Gronthos *et al.*, 2003a). In addition, the cell cycle progression from G1 to S phase may play a positive role in the proliferation and survival of BMSSCs-T *in vitro* and *in vivo*, because telomerase activity can increase the expression of G1-regulating genes, including those for cyclin D3, cyclin E1, E2F-4, and DP2, leading to an increased proliferation of BMSSCs-T (Gronthos *et al.*, 2003a).

Future directions

Although all the evidence so far accumulated strongly supports the notion that BMSSC-mediated bone formation is a promising approach for the repair of bone defects, there are some clear obstacles that need to be overcome for this procedure to lead to reliable therapies. Until now, the efforts of researchers in trying to understand mesenchymal stem cell populations have been limited to *ex vivo* expanded populations, grown in the presence of high serum levels. Typically, such culture systems induce cells to proliferate and differentiate, thereby greatly reducing the numbers of multi-potential populations over successive subcultures. Most of the cultured BMSSCs lose their stemness, at least partially. Therefore, finding optimal methods to maintain the stemness of BMSSCs, such as increasing the number of STRO-1 positive cells, during the *ex vivo* expansion will be critical for improving the capacity of bone formation. For example, telomerase expressing BMSSCs have been shown to display a significant increase in the population of STRO-1 positive cells, when compared with regular BMSSCs. Whether growth factor treatment induced osteogenesis is linked to the number of STRO-1 positive cells in BMSSC population remains to be elucidated.

The maintenance and regulation of normally quiescent stem cell populations has been shown to be tightly controlled by the local microenvironment, according to the requirements of the host tissue (Fuchs and Segre, 2000; Bianco and Robey, 2001). Identification of stem cell niches is extremely important because it will provide clues for understanding the characteristics of stem cells, and further manipulating the developmental potential of stem cells. The identification of the perivascular niche of BMSSCs represents significant progress in recent mesenchymal stem cell research (Owen and Friedenstein, 1988; Doherty *et al.*, 1998; Bianco *et al.*, 2001; Shi and Gronthos, 2003). BMSSCs could be retrieved efficiently by fluorescence activated cell sorting (FACS) from bone marrow aspirates, based on their high expression of the STRO-1 antigen (Gronthos *et al.*, 1994; Shi and Gronthos, 2003). STRO-1 is a marker of pre-osteogenic populations, where its expression is progressively lost following cell proliferation and differentiation into mature osteoblasts *in vitro* (Gronthos and Simmons, 1995; Stewart *et al.*, 1999; Dennis *et al.*, 2002). This is in accord with previous studies that have localized STRO-1 on large blood vessels, but not capillaries, in different adult tissues, such as brain, gut, heart, kidney, liver, lung, lymph node, muscle, and thymus (Bianco *et al.*, 2001). Therefore, STRO-1 appears to be an early marker of different mesenchymal stem cell populations that is also expressed by perivascular cells *in situ*. Now, it is possible to

isolate highly purified BMSSCs from bone marrow using STRO-1 in combination with an antibody directed to vascular cell adhesion molecule-1 (VCAM-1/CD106) (Gronthos *et al.*, 2003b). However, the practical value of such highly purified BMSSCs is a topic for future research.

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PART 3

Pharmaceutical Technology