BONE MARROW STROMAL CELLS: CHARACTERIZATION AND CLINICAL APPLICATION*

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*Dedicated to the memory of Professor Alexander J. Friedenstein, pioneer of bone marrow stromal cell research, inspiring teacher and friend.

ABSTRACT: The bone marrow stroma consists of a heterogeneous population of cells that provide the structural and physiological support for hematopoietic cells. Additionally, the bone marrow stroma contains cells with a stem-cell-like character that allows them to differentiate into bone, cartilage, adipocytes, and hematopoietic supporting tissues. Several experimental approaches have been used to characterize the development and functional nature of these cells *in vivo* and their differentiating potential *in vitro*. In vivo, presumptive osteogenic precursors have been identified by morphologic and immunohistochemical methods. In culture, the stromal cells can be separated from hematopoietic cells by their differential adhesion to tissue culture plastic and their prolonged proliferative potential. In cultures generated from single-cell suspensions of marrow, bone marrow stromal cells grow in colonies, each derived from a single precursor cell termed the colony-forming unit-fibroblast. Culture methods have been developed to expand marrow stromal cells derived from human, mouse, and other species. Under appropriate conditions, these cells are capable of forming new bone after *in vivo* transplantation. Various methods of cultivation and transplantation conditions have been studied and found to have substantial influence on the transplantation outcome. The finding that bone marrow stromal cells can be manipulated *in vitro* and subsequently form bone *in vivo* provides a powerful new model system for studying the basic biology of bone and for generating models for therapeutic strategies aimed at regenerating skeletal elements.

Key words. Bone marrow stromal cells, osteoprogenitor cells, transplantation, bone regeneration.

(I) *Introduction*

It is now well-appreciated that marrow is a complex tissue composed of two distinct but interdependent compartments: the hematopoietic system and the bone marrow stroma. Investigations into the cooperative interactions between these two compartments have shown that hematopoietic cells influence the activity of the stromal compartment, and in addition to serving as a mechanical support for differentiating hematopoietic cells, the bone marrow stroma also expresses cell-signaling factors that participate in the development of mature blood cells. A vast body of literature concerning hematopoietic cell transplantation exists; however, much less attention has been paid to transplantation of bone marrow stroma. Here, we review the development and functional nature of bone marrow stroma, and detail studies on the transplantation of bone marrow stromal cells (BMSCs) for the regeneration of skeletal elements.

(II) *The Bone Marrow Stroma* in vivo

"Stroma", in ancient Greek, means the physical substrate, or something upon which one rests or lies. Hematopoietic cells proliferate, differentiate, and mature upon a meshwork of cell processes and surfaces that together comprise the bone marrow stromal scaffold. In mammals, hematopoiesis is an extravascular process, and therefore is supported by an extravascular marrow stroma as well. The stroma includes all cell types that (1) are located between the outer surfaces of marrow blood vessels and the bone surfaces which encase the hematopoietic space and tissue, and (2) are not of the hematopoietic lineage, and these include marrow adipocytes, Westen-Bainton cells, bone-lining cells (inactive osteoblasts), and osteoblastic cells.

Westen-Bainton cells (Westen and Bainton, 1979; Bianco *et al.*, 1993) are specialized, hematopoiesis-supporting cells noted for, and defined by, the expression of high levels of membrane-associated alkaline phos-

phatase activity, the time-honored marker of osteogenic commitment in bone development. These cells are referred to by a variety of descriptive terms (reticular cells, adventitial reticular cells, and stromal fibroblasts). The diversity in nomenclature, together with the remarkably elusive morphology of the cells themselves, contributes to the vagueness of our concept of the marrow stroma as a tissue. Therefore, the eponymic designation (Westen-Bainton) and a functional phenotypic trait (alkaline phosphatase activity, ALP) best substitute for these terms. In the post-natal marrow, these cells provide an adventitial (outer) coating to the sinusoid wall on which they spread with no intervening physically continuous basement membrane. Extensive, elongated, and attenuated cell processes characterize their morphology ("reticular"). These cells express collagen types I and III, osteonectin, and are considered "fibroblastic" in nature. It should be noted, however, that the collagen fiber content is extremely sparse in the normal bone marrow.

In development, Westen-Bainton (WB) cells populate the primitive marrow spaces prior to the local appearance of hematopoietic cells. They originate from the primitive osteogenic periosteum of developing bones and co-migrate, along with ingrowing blood vessels, to the interior of developing bones, where they actively proliferate and provide the reservoir of immediate osteogenic precursors (proliferating pre-osteoblasts) for pre-natal osteogenesis (Bianco and Bonucci, 1991; Riminucci *et al.*, 1997). When the primitive, non-hematopoietic marrow is seeded with blood-borne hematopoietic stem cells, a close relationship is established between developing blood cells and WB (stromal) cells. Interestingly, endothelium-specific, carbohydrate-binding receptors termed selectins are expressed in stromal cells (PJ Simmons, personal communication), and likely mediate the physical interaction between maturing blood cells and an extravascular stroma. In the post-natal marrow *in situ*, WB cells generate and maintain the hematopoietic micro-environment. Indiscernible in routine histological material, and elusive even by electron microscopy, WB cells can be conveniently imaged *in situ* by ALP cytochemistry and confocal reflectance microscopy (Bianco and Boyde, 1989, 1993), which allows for documentation of their 3-D shape, mutual arrangement, and physical association with maturing hematopoietic cells. Their association with the adventitia of small arteries and the outer surfaces of sinusoids in the post-natal marrow reflects the developmental coupling of osteogenesis and vascularity.

Several pathological conditions in the bone/bone marrow organ can be seen as diseases of WB cells—that is, of osteogenic marrow stromal cells. In the developing marrow, cells identified as the major parathyroid-hormone-binding cell type *in vivo* (Rouleau *et al.*, 1988) are indistinguishable from WB cells. Interestingly, bone disease in human hyperparathyroidism is characterized by localized increases in WB cells, which result in the so-called "endosteal fibrosis" (Bianco and Bonucci, 1991). Likewise, in the McCune-Albright syndrome, where stimulation of the relevant signal transduction pathway is due to mutated, overactive Gsα, the fibrous dysplasia of bone results from an increase in number of WB cells (Riminucci *et al.*, 1997). Weston-Bainton cells also proliferate and mature to bone-forming cells responsible for osteosclerosis in cancer metastasis to the marrow (P Bianco, unpublished data).

In post-natal human marrow, Weston-Bainton cells demonstrate characteristics of pre-adipocytes (Bianco *et al.*, 1988). A progressive decline and eventual complete loss of ALP activity accompany their conversion to fat cells *in vivo*. Adipose conversion of ALP-positive WB cells is part of the general developmental program of the skeleton. An inverse relationship between ALP-positive WB cells and adipocytes can be demonstrated at any age or marrow site (Bianco *et al.*, 1993). Local peritrabecular adipose conversion of WB cells occurs in involutional osteoporosis. Thus, age-related changes in the size and functional properties of the WB cell compartment in the post-natal marrow represent an obvious direction for future investigation.

As pre-osteogenic and pre-adipogenic cells, WB cells are multipotential elements in the post-natal marrow. As an integral part of the hematopoietic micro-environment, they are also endowed with a phenotype and function that are differentiated in their own right. This emphasizes how retention of the potential for multiple differentiation (a "stem cell" characteristic) may be associated with differentiated function in the stromal system. This represents a major biological divergence from the hematopoietic system, from which the very concept of the stromal system was derived by analogy.

(III) *Bone Marrow Stromal Cells* in vitro

To date, the identity of the *in vitro* counterpart of the bone marrow stroma observed *in vivo* (Westen-Bainton cells) is not entirely clear. However, when bone marrow is plated *in vitro*, adherent cells of non-hematopoietic origin (*i.e.*, not macrophages) proliferate and exhibit many of the characteristics attributed to bone marrow stromal cells *in vivo* (Ashton *et al.*, 1985; Owen, 1988). In cultures generated from single-cell suspensions of marrow, colonies are formed, each derived from a single precursor cell termed the colony-forming-unit fibroblast (CFU-F) (Friedenstein *et al.*, 1978; Latsinik *et al.*, 1986), and the progeny of these CFU-F are what we define here as bone marrow stromal cells (BMSCs). When marrow cells are plated at higher densities, the colonies merge early and BMSCs grow as

monolayers. Due to a high proliferation potential, BMSCs can be expanded in culture to greater than a billion nucleated cells from a limited starting sample of 1 mL of a human bone marrow aspirate, or from the contents of the long bones of a guinea pig, rat, mouse, or rabbit (Friedenstein, 1980). Isolation of relatively pure BMSC cultures can be attained after subculture; however, the degree of purification may be dependent on the species and strain of animal used. Contamination with hematopoietic cells is higher in mouse BMSC strains than human BMSCs, and is diminished during consecutive passages *in vitro* (Krebsbach *et al.*, 1997; Kuznetsov *et al.*, 1997b). In addition to their fibroblast-like morphology, BMSCs share some, but not all, features with fibroblastic cells of other tissues, and lack the basic characteristics of endothelial cells and macrophages (Castro-Malaspina *et al.*, 1980; Fei *et al.*, 1990; Song *et al.*, 1995; Zhang *et al.*, 1995). After extended culture, BMSCs maintain the ability to form bone, cartilage, fibrous tissue, hematopoiesis-supporting reticular stroma and associated adipocytes, and perhaps muscle (Fig. 1). Thus, the BMSC population contains precursor cells capable of extensive proliferation and differentiation into several phenotypes (Chailakhyan *et al.*, 1978; Ashton *et al.*, 1980; Patt *et al.*, 1982; Owen, 1988; Bennett *et al.*, 1991).

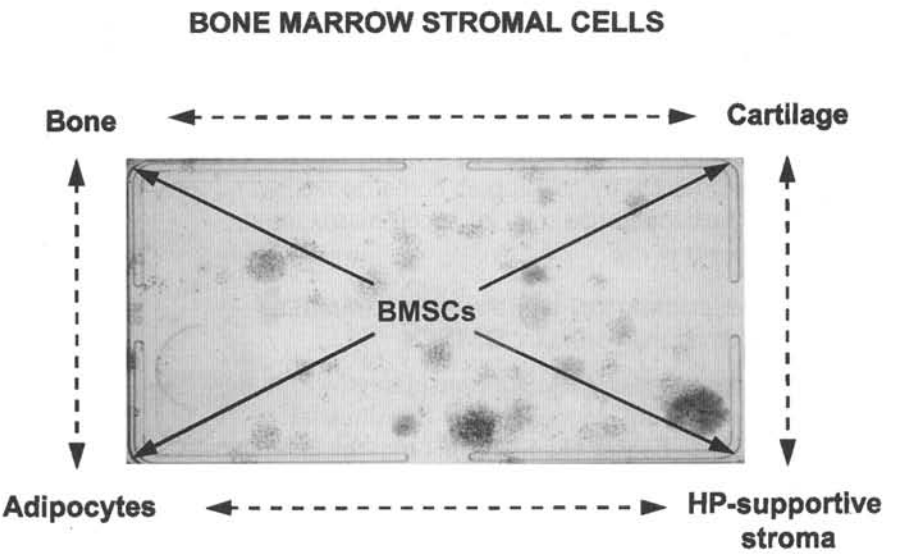

Figure 1. Pleuripotent nature of bone marrow stromal cells. *In vitro* and *in vivo* studies have demonstrated that multi-colony-derived strains of bone marrow stromal cells have the ability to form bone, cartilage, hematopoiesis-supportive stroma, and associated adipocytes. Additionally, in certain pathological conditions, *in vitro* studies have demonstrated that the phenotype expressed by a bone marrow stromal cell can be shifted from one type to another, demonstrating the extremely plastic nature of this cell population. As such, this population of cells is likely to play a major role in skeletal metabolism, in health and in disease states. The background of this Fig. shows individual BMSC colonies, each of which was derived from a single colony-forming-unit fibroblast.

Interest in BMSCs and their transplantation has increased because of the realization that BMSC strains can reform such a broad array of tissues. When transplanted under appropriate *in vivo* conditions, *ex vivo* expanded BMSCs regenerate bone and associated stromal structures and support hematopoiesis. This capacity has obvious clinical applications, especially since an alternative approach, induction of new bone by bone morphogenetic protein(s), would require large amounts of material and therefore may not be realistic in cases of massive defects (Yoshikawa *et al.*, 1996a).

Transplantation of BMSCs has distinct advantages over transplantation of the total marrow cell population. First, large numbers of BMSCs can be generated from a 1 mL marrow aspirate, thus avoiding the necessity for an additional surgery to harvest large amounts of autogenous marrow. For example, marrow harvested from long bones of a single rat provide enough cells for 2 or 3 successful bone-forming transplants, while BMSC cultures initiated by the same cell numbers provide enough cells for 50 transplants (Yoshikawa *et al.*, 1996a). Under appropriate conditions, BMSCs maintain their multipotential capability during prolonged cultivation and multiple passages *in vitro*, although it is true that a "stem-cell" character has never been definitively demonstrated (*i.e.*, the ability to self-renew continuously). However, the outcome of BMSC transplantation is strongly dependent on a number of cultivation and transplantation parameters, as well as on the BMSC species origin, as described below. Second, bone formation by transplanted BMSCs generally occurs more rapidly, and is more extensive than in transplants of total marrow cells (Goshima *et al.*, 1991b; Gundle *et al.*, 1995; Krzymanski and Wiktor-Jedrzejczak, 1996).

(IV) *Heterotopic Transplantation in Diffusion Chambers*

Diffusion chamber transplants have been used to test the osteogenic potential of both non-human and human cell sources. BMSCs can be transplanted *in vivo* within diffusion chambers made of Lucite rings bounded by two Millipore filters (0.45 mm pores). The chambers allow for the passive diffusion of nutrients and signaling molecules from the recipient, but do not elicit an immunologic response. The filters also prevent direct interaction between donor and recipient cells. Therefore, no vascu-

larization of the transplants occurs, and the tissues formed inside the chambers are entirely of donor origin. Although this closed system is quite useful for studying the differentiating capacities of transplanted cell populations, it is limited by its inability to characterize the requirements necessary to support hematopoiesis, and to identify the cooperative cell-cell or cell-matrix interactions that occur *in vivo*.

(A) Bone formation in diffusion chambers seeded with non-human BMSCs

Tissues formed by rabbit BMSCs transplanted within diffusion chambers are dependent on the dimensions of the chambers. In narrow chambers (0.1 mm thick), woven bone and fibrous tissue are formed. In wider chambers (2 mm thick), bone and fibrous tissue form on the periphery, while cartilaginous tissue often develops toward the middle of the chambers (Ashton *et al.*, 1980, 1984; Friedenstein *et al.*, 1987). These results suggest that factors influencing the differentiation of multipotential BMSCs include the diffusion of nutrients and oxygenation of cells. In deeper regions of the chambers, where diffusion of nutrients and oxygen supply would likely be reduced, cartilage is formed, whereas osteogenesis proceeds in regions neighboring the membranes of the diffusion chamber, where the surrounding vasculature contributes nutrients, cell-signaling factors, and oxygen. Rabbit BMSCs expanded *in vitro* for as many as 17 passages have been shown to retain their osteogenic potential (Miskarova *et al.*, 1970). This result is not influenced by the numbers of transplanted cells (in the range of 1×10^4 to 2×10^6 BMSCs *per* chamber) (Friedenstein *et al.*, 1987). Osteogenesis by guinea pig BMSCs is more dependent on the initial cell number within the chambers, with bone formed at high cell concentrations, but only fibrous tissue formed at lower concentrations (Chailakhyan and Lalykina, 1969). Neonatal pig BMSCs implanted at high concentrations in 2-mm-thick chambers consistently form bone, cartilage, and fibrous tissue 6-8 weeks after transplantation (Thomson *et al.*, 1993), while rat BMSCs are less osteogenic in the closed system and form bone only in 10% of the chambers at 8-12 weeks post-transplantation (Goshima *et al.*, 1991b).

(B) Bone formation in diffusion chambers seeded with human BMSCs

The osteogenic potential of human BMSCs has also been studied in diffusion chambers implanted intraperitoneally into immunocompromised mice. Interestingly, bone and cartilage formation was not detected 2-12 weeks after transplantation when the cells were cultured without dexamethasone (Ashton *et al.*, 1985; Haynesworth *et al.*, 1992; Gundle *et al.*, 1995). These results were not influenced by age or gender of the donors, or by the anatomical site from which the marrow was harvested (Haynesworth *et al.*, 1992). However, if human BMSCs were cultured in the continuous presence of dexamethasone (10^{-8} M) prior to transplantation, they produced osteogenic tissue with active osteoblasts, and cartilage deeper into the chamber (Gundle *et al.*, 1995).

(V) *Heterotopic Transplantation in Open Systems*

To study the cooperative cell-cell and cell-matrix interactions that occur in bone development and homeostasis of the adult skeleton, investigators have transplanted BMSCs into open systems (*i.e.*, no barriers between donor and host cells) such as under the kidney capsule of syngeneic animals. Transplantation under the kidney capsule allows the BMSCs to interact with a recipient environment that includes a rich vascular supply. As a result, more complex and physiologically relevant tissues can be formed than in diffusion chambers. A disadvantage of investigating the osteogenic potential of BMSCs in kidney capsule transplants is that in addition to being a technically difficult surgical procedure, only small tissue samples can be studied, and the number of samples analyzed *per* recipient animal is limited. To overcome these limitations, more recent studies have exploited the ability of immunocompromised mice to accept cell transplants from different strains and species of animals in a variety of anatomical sites that allow for multiple transplantations.

For open system transplants, the use and nature of the transplantation vehicle clearly are essential components for successful osteogenesis. Osteogenesis does not proceed when bone marrow suspensions are injected subcutaneously or intramuscularly, and when BMSCs are implanted as a cell pellet without a vehicle (Goshima *et al.*, 1991b; Yoshikawa *et al.*, 1996a), or when BMSCs are implanted into rapidly resorbed vehicles (Friedenstein *et al.*, 1981; Gorsheva *et al.*, 1981). Thus, it is evident that, in order to form bone, transplanted BMSCs require the presence of an organized substrate in which they can adhere and proliferate for periods long enough to ensure differentiation and osteogenesis. Of the vehicles used for BMSC transplantation to date, hydroxyapatite-based constructs have emerged as the most successful.

(A) Transplantation of non-human cells in open systems

Bone formation by rat BMSCs was studied in subcutaneous transplants in various types of hydroxyapatite (HA)-containing ceramics in the form of disks or cubes including HA coralline ceramic (Yoshikawa *et al.*, 1996a), hydroxyapatite/tricalcium phosphate (HA/TCP) (Goshima *et al.*, 1991b; Dennis *et al.*, 1992; Dennis and

Caplan, 1993), and self-setting porous HA cement (Yoshikawa *et al.*, 1996b). In most transplants, extensive bone formation developed. The histologic sequence of events leading to osteogenesis was reminiscent of that described during embryonic intramembranous bone formation. After one week, pores of the ceramic vehicle were filled with proliferating mesenchymal cells. At two weeks, osteoblast-like cells lined the surface of the ceramic and deposited small amounts of bone directly onto ceramic surfaces. By four weeks, new bone covered most of the pore surfaces and was followed by bone remodeling in close association with the ingrowing vasculature. At eight weeks, marrow cavities containing a substantial amount of hematopoietic cells were formed in conjunction with the new bone (Goshima *et al.*, 1991b). Bone formation started on the surface of the ceramic and advanced toward the center of the pore (Yoshikawa *et al.*, 1996a). In most transplants, no cartilage was found, though chondrocytes could be occasionally observed in avascular pores (Goshima *et al.*, 1991b; Dennis *et al.*, 1992; Dennis and Caplan, 1993). Interestingly, ceramics pretreated with fibronectin or laminin retained twice the number of BMSCs than non-treated ones, and led to more rapid osteogenesis than non-treated vehicles (2 weeks *vs.* 4 weeks). In addition, more transplants in HA/TCP ceramic were positive for bone than in coral-based ceramic (Dennis *et al.*, 1992; Dennis and Caplan, 1993). By means of a more typical tissue engineering strategy, it was shown that bone formation could be accelerated if rat BMSCs were precultured within HA-containing vehicles for two weeks prior to transplantation (Yoshikawa *et al.*, 1996a). This approach could be promising for clinical applications of autogenous BMSCs and deserves further investigation for human cells.

Bone and bone marrow were formed by rat BMSCs that were cultured for four weeks on a type I collagen matrix prior to transplantation. In these experiments, the cells had an opportunity to synthesize a matrix within the vehicle. If BMSCs had been cultured on the collagen matrix for only two days, the collagen matrix was rapidly resorbed, no bone formed after transplantation, and fat tissue developed instead (Mizuno *et al.*, 1997). These data emphasize that, for successful bone formation by BMSCs that have been cultured *in vitro*, the transplanted vehicle should be sustained *in vivo* for at least several weeks to allow for adequate proliferation and differentiation necessary for the initiation of osteogenesis. If the vehicle is resorbed prior to these events, such as is the case for certain types of collagen matrices, bone formation will not ensue. Some success with resorbable vehicles has been reported for transplants of rat BMSCs in poly(DL-lactic-co-glycolic acid) foam. After a one-week *in vitro* culture period in which rat BMSCs were able to proliferate and establish an extracellular matrix within poly(DL-lactic-co-glycolic acid) foams, transplants were placed into the rat mesentery (Ishaug-Riley *et al.*, 1997). Ectopic bone was formed as early as one week post-transplantation; however, the mineralized tissue penetrated only about 200 to 400 microns into the polymer implant. Considered collectively, these data suggest that the successful use of rapidly resorbing vehicles may require an *in vitro* growth period to allow BMSCs to organize an extracellular matrix so that a BMSC network primed for osteogenesis will be implanted.

Mouse BMSCs have been transplanted in collagen sponges under the kidney capsule of syngeneic recipients. Ossicles containing bone marrow were developed in most transplants by 4-5 weeks, and they were maintained for at least five months (Grosheva *et al.*, 1981; Friedenstein *et al.*, 1982; Kuznetsov *et al.*, 1989). When mouse BMSCs were transplanted subcutaneously into immunocompromised recipients, they formed bone complete with hematopoietic tissue in all vehicles tested: collagen sponges, collagen matrices, polyvinyl sponges, HA/TCP blocks, and HA/TCP powder (Krebsbach *et al.*, 1997). In collagen sponges, the new bone appeared as early as two weeks post-transplantation. At four weeks, a cortical-like bone capsule developed surrounding an area with active hematopoiesis. The bone marrow tissue included reticular and adipocytic stroma, and hematopoietic cells of all lineages and stages of maturation, including colony-forming cells, in numbers equivalent to those of skeletal marrow. Both bone and hematopoietic tissue showed no signs of degeneration or senescence for at least 14 weeks post-transplantation (Krebsbach *et al.*, 1997) and, thus, could be considered self-maintained.

Rabbit BMSCs were transplanted autogenously under the kidney capsule inside Plexiglas rings. Heterotopic ossicles were formed in all 45-90-day-old transplants. Interestingly, the nature of the associated marrow tissue mirrored the original bone marrow from which BMSC cultures had been initiated. BMSCs cultured from cellular (red) marrow produced a stroma with numerous hematopoietic foci, whereas those cultured from a hypocellular (yellow) marrow produced a stroma replete with fat cells (Patt *et al.*, 1982). These important data imply that rabbit BMSCs "remember" their origin during numerous passages *in vitro* and after *in vivo* implantation, and recreate the structure of their original tissue. It would be interesting to study whether the same phenomenon can be demonstrated for BMSCs of other species, including human.

In open system transplants, however, it is necessary to determine the origin (donor *vs.* recipient) of tissues formed, since it is conceivable that transplanted cells could induce undifferentiated mesenchymal cells present at the site of transplantation to form bone. Studies

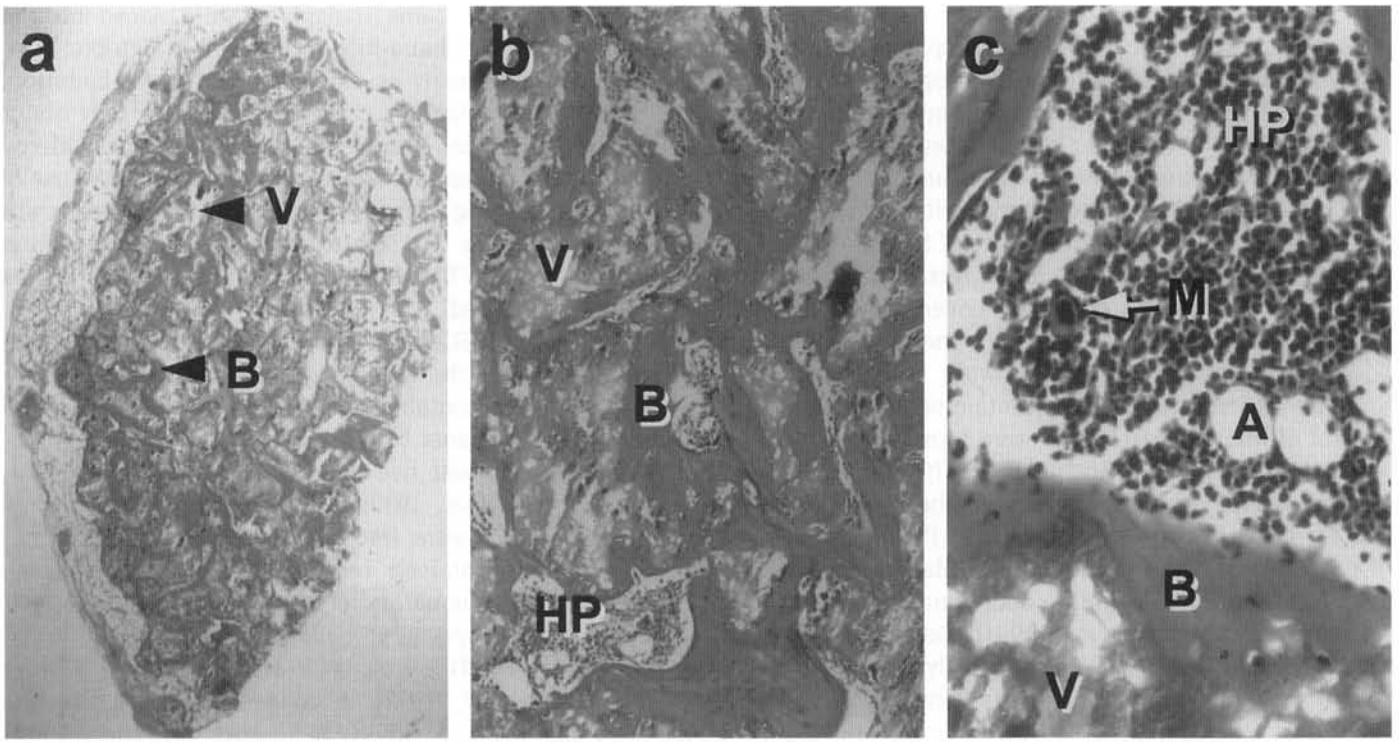

Figure 2. Demonstration of the formation of a bone/bone marrow ectopic ossicle by transplanted bone marrow stromal cells. (a) When *ex vivo* expanded human bone marrow stromal cells are attached to ceramic particles or Collagraft™ as a vehicle (V) and transplanted into a subcutaneous site in immunocompromised mice, ectopic bone (B) is formed within 4-6 weeks. (b) This ectopic ossicle exhibits extensive bone formation (B) by osteoblastic cells in close association with the vehicle particles (V). (c) Further, complete hematopoiesis (HP) *including megakaryocytes (M) and adipocytes (A) is supported in close association with the newly formed bone (B).*

using chromosomal markers, mouse strain-specific antiserum, species-specific antibodies, and reverse transplantation have shown that in heterotopic transplants of either rodent marrow cells or BMSCs, bone-forming cells are of donor origin. By similar criteria, hematopoietic cells and macrophages originate from the recipient (Friedenstein *et al.*, 1968, 1978; Hotta *et al.*, 1983). In transplants of BMSCs derived from transgenic mice bearing a type I collagen-chloramphenicol acetyltransferase (CAT) reporter gene, cells within the newly formed bone displayed CAT activity up to at least 14 weeks post-transplantation (Krebsbach *et al.*, 1997), providing yet another method to follow the fate of the transplanted cells. In one report, transplants of quail marrow into nude mice showed a biphasic switch in the origin of new bone-forming cells (Goshima *et al.*, 1991a). After three to four weeks *in vivo*, osteocytes were of donor origin, but were later identified to be of host origin by eight to 12 weeks post-transplantation, by means of a specific nuclear marker. Although this switch in the origin of bone-forming cells has not been observed with rodent or human cells, it is certainly conceivable that as the newly formed tissue matures, mesenchymal cells of recipient origin in the microenvironment of the transplant may be induced to differentiate into functional osteoblasts.

(B) Transplantation of Human Cells

Human BMSCs transplanted subcutaneously into immunocompromised mice in HA or HA/TCP ceramic blocks (in the form of disks or cubes) consistently formed bone four to eight weeks post-transplantation. Bone was deposited directly in apposition to the ceramic surface and developed from the surface toward the center of the pores. At 6-8 weeks, bone remodeling and formation of marrow occurred, and no evidence of cartilaginous tissue was reported to be found in any of the grafts (Ohgushi and Okumura, 1990; Haynesworth *et al.*, 1992; Gundle *et al.*, 1995; Martin *et al.*, 1997). Systematic studies that used various transplantation vehicles demonstrated that human BMSCs are more dependent on the nature of the vehicles than are mouse BMSCs. Human BMSCs consistently form bone in vehicles containing HA/TCP ceramics in the form of blocks, powder, or Collagraft™ (mixture of ceramic powder with bovine collagen type I). With HA/TCP powder and Collagraft™, more extensive bone formation was observed than with HA/TCP blocks, and even more bone was formed when powder particles were held together with a secondary vehicle, such as a fibrin clot. In collagen sponges, human BMSCs formed bone only sporadically and only after cultivation with dexa-

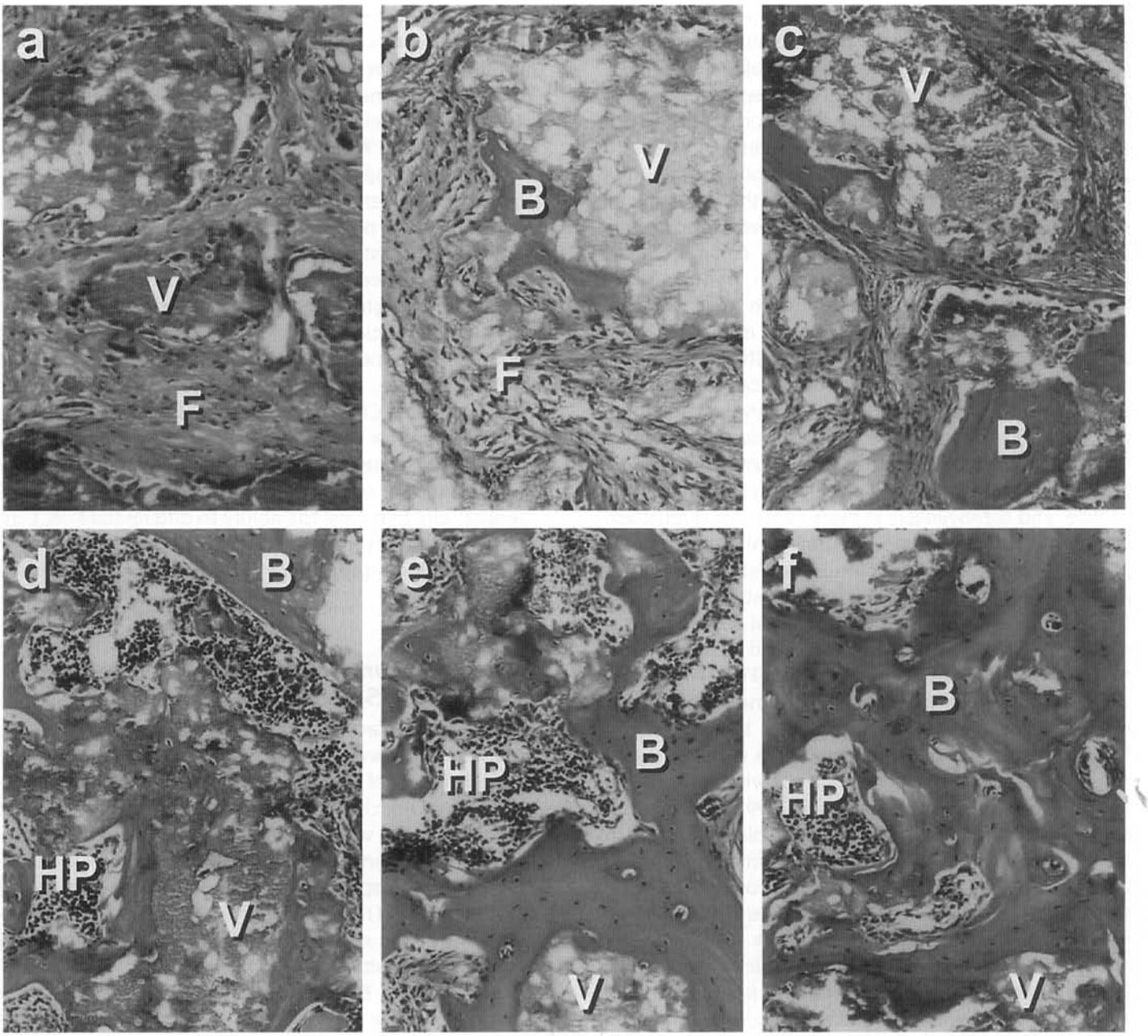

Figure 3. Heterogeneity of the osteogenic capacity of clonally derived human bone marrow stromal cells demonstrated by *in vivo* transplantation. Individual clones, arising from the proliferation of a single CFU-F, were isolated from low-density cultures of human bone marrow cell suspensions and expanded in culture in order to generate enough cells for transplantation. Cells were attached to ceramic particles or adsorbed to Collagraft™ as vehicles (V), and transplanted subcutaneously into immunocompromised mice. Transplants were harvested 6 weeks later and histologically analyzed. In some clones, only fibrous tissue (F) was formed (a), while in other clones fibrous tissue (F) along with low levels of bone (B) were formed (b). In other clones, larger amounts of bone were formed, but hematopoiesis was absent (c). Approximately 24% of the clones formed bone and supported hematopoiesis (HP) (d, e). In some cases (e), the clone was entirely equivalent to a multi-colony-derived strain (f) in the amount of bone and HP formed in the ossicle.

methasone. No bone formation was observed in the polyvinyl sponges, poly(L-lactic acid), and human demineralized bone matrix (Krebsbach *et al.*, 1997). Likewise, human BMSCs formed bone when transplanted into HA cubes, and loose connective tissue when transplanted into collagen sponges (Martin *et al.*, 1997). Transplants with extensive bone formation always demonstrated abundant hematopoietic tissue located close to the newly formed bone (Krebsbach *et al.*, 1997; Kuznetsov *et al.*, 1997b). It should be noted that HA/TCP blocks (cubes or disks) used in the majority of these studies proved to be less satisfactory for transplantation

of human BMSCs than either HA/TCP powder (with or without secondary vehicle) or Collagraft™. There is no reliable way to load cells into ceramic blocks (Dennis *et al.*, 1992). Whatever method is used (negative pressure, direct cell suction, etc.), only a small portion of the cell suspension can find its way into the vehicle, mostly into peripheral pores. As a consequence, bone formation is usually limited to the periphery of the transplants. Moreover, there is no simple way to determine how many cells were actually loaded into the ceramic block. Specially designed experiments with ^{3}H-thymidine-labeled cell quantitation have shown that between 45,000 and 65,000 cells are loaded *per* 27 mm^3 block when the cell suspension contains 5 x 10^6 cells/mL (Dennis *et al.*, 1992). In contrast, ceramic powder can be quantitatively loaded by slow rotation of the cell/vehicle mixture; this method is based on the high adhesive properties of BMSCs. As a result, more than 80% of total cells can be loaded, cells are distributed more evenly throughout the vehicle, and osteogenesis is more extensive (Krebsbach *et al.*, 1997). In transplants of human BMSCs in ceramic powder, bone and hematopoietic tissue showed no signs of degeneration or senescence for at least 45 weeks post-transplantation (Kuznetsov *et al.*, 1997b). Consequently, the newly formed heterotopic bone and hematopoietic microenvironment are self-maintained (Fig. 2).

In 4-8-week-old transplants of human BMSCs into immunocompromised mice or rats, three different human-specific antibodies reacted with osteocytes and osteoblasts of the new bone, but not with surrounding recipient tissues (Haynesworth *et al.*, 1992; Krebsbach *et al.*, 1997; Martin *et al.*, 1997). In transplants of human BMSCs, cells of the new bone were immunodetected with antibodies raised against human osteonectin and expressed human-specific *alu* sequences for at least 45 weeks post-transplantation (Kuznetsov *et al.*, 1997b). These data indicate that, in heterotopic transplants of human BMSCs into an open system, as in transplants of rodent BMSCs, new bone maintains the donor origin. On the contrary, hematopoietic cells associated with the new bone are of recipient origin (Kuznetsov *et al.*, 1997b).

(C) Transplantation of clonal BMSC strains

As previously mentioned, when marrow cells are plated at low cell densities, BMSCs grow as discrete colonies, each formed by proliferation of a single CFU-F (Friedenstein *et al.*, 1978; Latsinik *et al.*, 1986). The studies reviewed above have used multi-colony-derived strains consisting of descendants of multiple BMSC colonies—*i.e.*, many CFU-Fs. However, to analyze the natural heterogeneity of a CFU-F population, one must study descendants of individual CFU-Fs. About 20% of the colonies formed bone when individual colonies of mouse or guinea pig BMSCs were transplanted (Chailakhyan *et al.*, 1978). When rabbit single-colony-derived BMSC strains were transplanted into diffusion chambers, either autogenously or into immunocompromised mice, bone was formed by 48.3% (Gerasimov *et al.*, 1986; Friedenstein *et al.*, 1987) and 36.8% of the strains (Bennett *et al.*, 1991), respectively.

Human single-colony-derived BMSC strains from four donors were transplanted into immunodeficient mice in HA/TCP powder and Collagraft. After eight weeks, 20 out of 34 strains (58.8%) formed bone, and the remainder formed only fibrous tissue. Among the 20 positive strains, eight formed extensive bone accompanied by hematopoietic tissue (23.5%), and 12 formed less abundant bone without hematopoiesis (35.3%) (Kuznetsov *et al.*, 1997b) (Fig. 3). These data demonstrate that individual CFU-Fs are capable of forming both bone and a supportive hematopoietic microenvironment. They also demonstrate that the CFU-F population is heterogeneous and that only a fraction of it consists of multipotential cells that have the ability to differentiate sequentially into several tissues, including bone and hematopoiesis-supportive stroma with associated adipocytes.

(VI) *Factors Influencing* Bone Formation *in Open System Transplants of* BMSCs

Many of the factors that influence osteogenesis by BMSCs have been discussed above and include the species origin of BMSCs, the composition of the transplantation vehicle, and the method by which cells are loaded into the vehicle. It is likely, however, that several other factors participate to accelerate or impede osteogenesis by transplanted BMSCs. Among these potential modifiers are: (1) the optimum *in vitro* cell expansion conditions, (2) the cell seeding density, and (3) the ideal vehicle composition. Other factors—such as the cryopreservation of BMSCs before transplantation and the origin of marrow (age and gender of donor, skeletal location, method of marrow extraction)—show no obvious correlation with frequency or extent of bone formation (Goshima *et al.*, 1991b; Haynesworth *et al.*, 1992; Bruder *et al.*, 1997a; Krebsbach *et al.*, 1997). The number of *in vitro* passages and population doublings of the BMSCs before transplantation did not affect bone formation as long as the cells retained their proliferative status. However, mouse BMSCs of the 13th passage that had ceased to proliferate *in vitro* did not form bone *in vivo* (Krebsbach *et al.*, 1997). Rat BMSCs formed bone for up to 18 passages. However, osteogenesis was delayed and the rate of bone formation declined with increasing passage number (Goshima *et al.*, 1991a). In an extensive *in vitro* study, it was demonstrated that cultured BMSCs derived from human iliac crest aspirates average about 38 population

doublings before becoming senescent, and despite reaching a point of senescence, the cells remained capable of osteogenesis (Bruder *et al.*, 1997b).

To date, the dependence of *in vivo* bone formation on the number of transplanted BMSCs has not been systematically studied. When mouse BMSCs are transplanted under the kidney capsule in collagen sponges, ossicles containing bone marrow are formed in most of the transplants containing from 1 x 10^5 to 2 x 10^6 BMSCs, but not in transplants containing fewer than 0.5 x 10^5 BMSCs (Grosheva *et al.*, 1981; Friedenstein *et al.*, 1982). Only those ceramics (27 mm^3 cubes) that retain greater than 3 x 10^4 rat BMSCs *per* transplant consistently form bone (Dennis *et al.*, 1992; Dennis and Caplan, 1993). Cell number may not influence the extent of bone formation throughout the range of 1.5 to 16 x 10^6 mouse or human BMSCs *per* transplant (20-100 mm^3 vehicle volume) (Krebsbach *et al.*, 1997). However, in light of the above-mentioned studies, these transplants likely exceeded the threshold necessary for osteogenesis. Thus, it is evident that the minimum number of BMSCs necessary for bone formation after implantation is yet to be determined.

Medium composition can also significantly affect bone formation by transplanted BMSCs. Specifically, selected lots of serum appear to be particularly important for growth of BMSCs from rodent species; however, the factors that are present in various amounts from one lot of serum to another have not been delineated. For mouse BMSCs, the presence of dexamethasone in culture medium did not influence the extent of bone formation (Krebsbach *et al.*, 1997). Similar observations were noted with human BMSCs transplanted into HA/TCP-containing vehicles (Gundle *et al.*, 1995; Krebsbach *et al.*, 1997; Kuznetsov *et al.*, 1997b). However, human BMSCs transplanted into diffusion chambers or collagen sponges formed bone only after cultivation in medium that included dexamethasone (Gundle *et al.*, 1995; Krebsbach *et al.*, 1997). In addition, when low numbers of human BMSCs were transplanted (2.5 x 10^5 *per* 64 mm^3 HA ceramic cube), cultivation with dexamethasone increased the extent of bone formation (Martin *et al.*, 1997). Rat BMSCs transplanted into coralline HA ceramic cubes formed bone only after cultivation with dexamethasone (Yoshikawa *et al.*, 1996a).

The effects of several growth factors on bone formation by transplanted BMSCs have also been studied. PDGF-BB (5 ng/mL, present for 48 hrs before transplantation) did not change the *in vivo* bone formation by rat BMSCs, while TGF-β decreased bone formation in 50% of the experiments under similar conditions (Cassiede *et al.*, 1996). Six-day pretreatment with BMP-2 (50 ng/mL) did not stimulate bone formation by rat BMSCs, while pretreatment with bFGF (2.5 ng/mL) enhanced bone formation, which was further enhanced by a combination of bFGF with BMP-2 (Hanada *et al.*, 1997). Human BMSCs grown in the presence of FGF-2 (1 and 10 ng/mL) yielded significantly greater bone formation than those grown without it, while other growth factors, including PDGF, EGF, IGF-I, TGF- β1, and GH, did not affect the extent of bone formation (Martin *et al.*, 1997).

(VII) *Potential Clinical Applications*

(A) Orthotopic transplantation of BMSCs

Reconstruction of defective bone by means of graft material, composed of mixtures of autogenous marrow and a variety of different vehicles, is a widely used procedure in orthopedics and oral surgery, the goal of which is to restore the functional integrity of bone. Bone grafting is a dynamic phenomenon: A successful graft heals, becomes incorporated, revascularizes, and eventually assumes the desired form and function. The ideal grafting material fulfills three physiologic functions: First, it provides a source of bone-forming cells; second, it induces cells (either grafted or indigenous) to form bone, a process called osteoinduction; and finally, it provides a scaffold for new bone deposition, a process known as osteoconduction.

(B) Animal studies

Determining the requirements for ectopic osteogenesis in *in vivo* transplants of BMSCs is an important first step in understanding how these populations of cells may be used for future regenerative therapies. However, the microenvironment of a subcutaneous implant site is far different from that of a skeletal site in need of bone regeneration. There are few studies that have investigated the regenerative potential of BMSCs in orthotopic transplants, that is, in skeletal defects. In orthotopic conditions, the differentiation of transplanted BMSCs is influenced by both the transplantation site and vehicle composition. Mouse BMSCs transplanted *via* collagen sponges into a 5-mm calvarial defect formed bone as early as two weeks post-transplantation. At 4-5 weeks, the new bone completely covered the defect and could be distinguished from pre-existing bone by a large cavity filled with hematopoietic marrow. When transplanted BMSCs were derived from transgenic mice harboring a collagen type I-CAT transgene, cells of the new bone were positive for CAT, thus demonstrating donor origin of the new bone. The defect remained unhealed if empty vehicles or vehicles filled with mouse spleen stromal cells were transplanted (Mankani *et al.*, 1997a; Krebsbach *et al.*, 1998).

Autogenous rabbit BMSCs dispersed in a bovine type I collagen gel have been transplanted into a large (3 x 6 mm), full-thickness defect in the weight-bearing sur-

face of the medial femoral condyle. As early as two weeks post-transplantation, the transplanted cells differentiated into chondrocytes in the upper half of the defect; the deeper half was filled with newly formed cancellous bone. At 24 weeks, the subchondral bone was completely repaired, without loss of overlying articular cartilage. The cartilage surface remained smooth, although the cartilage became thin. In cell-free controls, there was substantially less repair. Mechanically, the reparative tissue was stiffer than in cell-free controls, but less stiff than normal cartilage (Wakitani *et al.*, 1994).

Autogenous canine BMSCs were loaded into fibronectin-coated HA/TCP cylinders and transplanted into 21-mm-long segmental femoral mid-diaphyseal defects. Rapid bone formation occurred at all host-implant interfaces, resulting in union of all animals by eight weeks. A substantial collar of bone developed surrounding the implant itself; after 12 weeks, the callus began to remodel to the original dimension of the implant. Animals whose defects were not filled formed a fibrous non-union. If cell-free vehicles were implanted, bony ingrowth was limited to the interface, and the bulk of the implant was devoid of bone. Cancellous bone from the iliac crest was also transplanted to some defects; these transplants were radiographically equivalent to the BMSC-based ones (Bruder *et al.*, 1998b,c).

(C) Human studies

In humans, blood and bone marrow are the most frequently transplanted materials (Allard *et al.*, 1987; Pedrosa *et al.*, 1989; Aglietti *et al.*, 1991; Eggers and Meeder, 1994; Friend *et al.*, 1995; Olsen *et al.*, 1995; Arrington *et al.*, 1996; Bradbury *et al.*, 1996). Fresh autogenous bone grafts are preferred in orthotopic procedures because of their compatibility and efficacy. In addition, autogenous grafts are able to trigger direct osteogenesis by bone cells. Autogenous iliac bone is considered to have the highest osteogenic potential, and indeed, the osteogenic potential has been shown to reside within the marrow stromal compartment. This is supported by the clinical observation that autogenous bone impregnated with red marrow has a beneficial effect on osteogenesis. However, the amount and quality of implanted bone needed limit the use of autogenous bone alone. Large defects may require a larger amount of bone than is available for harvesting (Arrington *et al.*, 1996). In addition, if the quality of bone is poor, such as in certain disease states, the autogenous bone may not be appropriate for engraftment. These limitations point to the real need for the development of procedures that use *ex vivo* expanded BMSCs.

Experiments where human BMSCs are transplanted orthotopically into animal recipients represent an intermediate step between ectopic animal transplantation and orthotopic transplantation into human patients. Human BMSCs of the 2nd passage were loaded into fibronectin-coated HA/TCP cylinders and transplanted into 8 mm central diaphyseal defects of athymic rats. By 12 weeks, 47% of implant volume was filled with bone of human origin; control transplants of empty vehicles contained 27% bone. Also, stiffness and failure load of BMSC-loaded implants was significantly greater than those of cell-free implants (Bruder *et al.*, 1997c, 1998c).

There are currently few studies that have attempted the clinical use of *ex vivo* expanded human BMSCs. Autogenous human BMSCs of various passages have been loaded into demineralized allogenic cancellous bone matrix and transplanted into long-lasting bone defects of 20 patients. In these studies, there were seven patients with non-union fractures, eight with pseudoarthroses, and five with long bone defects. Results from 15 patients were followed for up to two years. All 15 patients demonstrated union and filling of the defects, which was considerably faster and more efficient than following other treatments (Osepjan *et al.*, 1987). Composite grafts of fresh autogenous marrow, cultured autogenous BMSCs, and allogeneic lyophilized bone fragments have also been transplanted into mandibular defects immediately after surgery. After one month, bone defects showed significant bone ingrowth; after three months, they were completely filled with well-mineralized bone in most patients. In control patients transplanted with marrow cells plus bone fragments, results were similar but were delayed by approximately three months. In historical controls where no transplantation had been performed, the healing was even slower, and sometimes incomplete by 12 months (Krzymanski and Wiktor-Jedrzejczak, 1996).

(D) Vascularized bone grafts

There are a number of clinical situations in which the recipient site may not be amenable to direct transplantation of BMSCs in combination with appropriate vehicles, and bone healing may not be optimal. For example, following high-dose radiation prior to surgery for the removal of cancerous tissue, or in cases of persistent deep infections, the recipient site can display a high degree of morbidity and compromised vascularization. This site morbidity may have a profound influence on the rate at which transplants integrate, and may ultimately lead to transplant failure. For this reason, the use of vascularized bone grafts has been developed whereby segments of normal bone with intact vasculature are removed from a donor site and placed into a morbid recipient site to establish anastomosis with intact vasculature at the margins (Storm *et al.*, 1996).

A preliminary study in mice has shown that *ex vivo* expanded BMSCs can be used to generate bone sur-

rounding an isolated artery and vein. BMSCs in vehicle were wrapped around the internal carotid artery and inferior jugular vein, and further encircled with an expanded polytetrafluoroethylene membrane (Goretex™) to prevent collateral vascularization. After eight weeks, the transplant contained cortical and cancellous bone fully perfused by the artery and vein. This technique shows a great deal of promise for reconstructive surgery such that a vascularized bone graft can be generated within the patient's own body and then relocated to a recipient site as needed (Mankani *et al.*, 1997b).

(E) Systemic distribution of BMSCs

The methodologies described above are very applicable to the restoration of segmental bone defects. However, in cases of generalized bone loss such as in a variety of skeletal diseases including osteoporosis, osteogenesis imperfecta, etc., it is not feasible to consider direct transplantation into all affected bones. In these cases, it would be desirable to inject BMSCs for distribution *via* the circulatory system such that they would be widely dispersed, and stimulated in some fashion to regenerate new (and/or better) bone.

Injection of marrow stromal cells has been attempted in both animal models and in humans, with limited success. There are numerous reports that stromal cells present in the cell population injected during bone marrow transplantation procedures do not survive their travels in the circulation, and do not repopulate bone or any other tissue (Friedenstein and Kuralesova, 1971; Friedenstein *et al.*, 1978; Wilson *et al.*, 1978; Golde *et al.*, 1980; Chertkov *et al.*, 1985; Laver *et al.*, 1987; Ma *et al.*, 1987; Simmons *et al.*, 1987; Raskind *et al.*, 1988; Athanasou *et al.*, 1990; Ribera *et al.*, 1990; Agematsu and Nakahori, 1991; Santucci *et al.*, 1992). However, when partially purified bone marrow stromal cells isolated from transgenic mice expressing a human mini-gene for collagen type I as a marker were injected into irradiated mice, polymerase chain-reaction assays for the collagen mini-gene indicated that, after 1-5 months, the donor cells accounted for 1.5-12% of the cells in bone, cartilage, and lung in addition to marrow and spleen (Pereira *et al.*, 1995). In other studies, an immortalized marrow stromal cell line engineered to express β-galactosidase was also found in bone after injection (Dahir *et al.*, 1996). These results suggest that BMSCs expanded in culture may serve as long-lasting precursors of bone, cartilage, and lung. However, it should be noted that in the first study, the marrow stromal cells were likely contaminated with macrophages which may engraft easily, and in the second case, the cells used were immortalized, which may represent a selective advantage to survival in the circulatory system compared with non-immortalized, normal cells. In a recent report, Pereira and colleagues showed that infusion of marrow stromal cells from wild-type mice could partially rescue a phenotype of fragile bones resembling osteogenesis imperfecta (Pereira *et al.*, 1998). DNA from the donor marrow stromal cells was detected consistently in marrow, bone, cartilage, and lung either one or 2.5 months after the infusions, suggesting that marrow stromal cells or related cells in marrow may serve as a source for continual renewal of cells in a number of nonhematopoietic tissues.

The route of injection may also influence the survival of injected BMSCs (Mankani *et al.*, 1997b). If injected *via* the tail vein, BMSCs were removed primarily by the lungs, and if injected *via* the left ventricle, the distribution was somewhat broader but still did not result in localization of the BMSCs to bone or bone marrow. Only when the cells were injected into the abdominal carotid arteries were significant levels of BMSCs identified in bone marrow, in addition to skin, muscle, and spleen. However, it has not yet been demonstrated that these cells survive long term and actually engraft.

Several human patients have been transfused with *ex vivo* expanded BMSCs (Lazarus *et al.*, 1995), although the results concerning engraftment of these cells within these patients are not yet available. Taken together, the results of these experiments indicate that the dissemination of BMSCs *via* systemic injection is not routine, and to date, specific homing of BMSCs to bone has not been achieved by means of either normal or immortalized cell lines. From these studies, it appears that it may be necessary to provide BMSCs with a homing device—*e.g.*, a cell-surface molecule that would enable to BMSCs to remain resident within the bone marrow, either through transient or stable genetic engineering, or through the treatment of the cells with factors that will enable them to produce the required molecules.

(F) BMSCs and gene therapy

Based on the fact that transplanted BMSCs form a stable ossicle containing bone and stromal cells of donor origin that is self-maintained for long periods of time, BMSCs appear to be good candidates for gene therapy. During *ex vitro* expansion, BMSCs may be genetically manipulated to produce a desired cellular product. Generation of an ectopic bone ossicle or pedicled bone graft in a subcutaneous site by means of engineered BMSCs could be envisioned as an *in vivo* "mini-pump", generating the desired factor and releasing it into the circulation. Such an ectopic ossicle would have the advantage of allowing for easy removal should the "therapy" supplied by the ossicle no longer be required. Furthermore, new methods are being developed to correct genetic mutations by a number of strategies including hammerhead ribozymes (Sakai *et al.*, 1996; Grassi *et al.*, 1997). It can be envisioned that autogenous BMSCs could be engineered to correct a

defective gene and be systemically distributed (when conditions for this procedure have been established) in order to re-establish a normal bone/marrow microenvironment.

Currently, there is a variety of methods that utilize either retroviral or adenoviral vectors that mediate stable or transient transfection of a target cell population. While the success of these types of transfections is routine *in vitro*, reports of their successful utilization *in vivo* to bring about gene transfer has been sporadic at best. Recently, it has been demonstrated that normal human BMSCs can be transfected with adenovirus coupled to β-galactosidase as a marker (MF Young, unpublished results). Furthermore, transplants made by cells expressing lacZ and IL-3 have been generated, and IL-3 was detected in the circulation of the animals for up to 12 weeks after transplantation (Allay *et al.*, 1997).

(G) Animal models of human bone disorders

The observation that BMSCs are osteogenic precursors capable of recreating the architecture of their original tissue gave rise to the hypothesis that transplanted BMSCs may recapitulate the pathophysiology of skeletal disease, thus creating an *in vivo* model of skeletal disorders. So far, this assumption has been proved true for the McCune-Albright Syndrome (MAS). McCune-Albright Syndrome is a non-inherited genetic disease caused by activating missense mutations of the gene encoding the α subunit of the stimulating G protein. MAS is a mosaic disorder whose manifestations include endocrinopathies, skin hyperpigmentation, and polyostotic fibrous dysplasia of bone. When BMSC colonies from MAS patients were cultured, they were found to be of two types: normal (cells contained two normal Gsα alleles) and mutant (one normal allele and one with mutation). Subcutaneous transplantation of normal BMSCs into immunocompromised mice resulted in the formation of normal ossicles, while transplantation of mutant cells alone led to the loss of transplanted cells with no ossicle formation. However, transplantation of a mixture of normal and mutant cells reproduced an abnormal ectopic ossicle recapitulating many features of human fibrous dysplasia (Bianco *et al.*, 1998). These data provide additional insight into the pathophysiology of fibrous dysplasia in MAS and offer a novel approach to the study of the disease and potential therapeutic interventions. More generally, transplantation of pathologic BMSCs *in vivo* may provide a promising strategy for generating animal models of other human bone disorders.

(VIII) *Future Directions*

While our understanding of the nature of BMSCs has been greatly expanded during recent years, it is clear that there is still much to be learned about these important mediators of skeletal metabolism, and that their further characterization is crucial for their clinical application. Questions that remain unanswered include: (1) What is the *in vivo* identity of the CFU-F, the progeny of which we have partially characterized as BMSCs *in vitro*? (2) How do CFU-F and their progeny participate in the processes of normal skeletal metabolism, and how are these processes changed in disease states? and (3) What are the regulatory factors that influence the activity of CFU-F and their progeny to determine which phenotype they will express, both *in vivo* and *in vitro*?

If these questions are to be answered, it is clear that better identification of CFU-Fs and BMSCs must be made, both *in vivo* and *in vitro*. To date, there is only a handful of markers (as identified by monoclonal antibodies) that appear to be specific for these cells (Akasaka *et al.*, 1991; Simmons and Torok-Storb, 1991; Benayahu *et al.*, 1995; Masuhara *et al.*, 1995; Bruder *et al.*, 1997a). For the most part, the antigens recognized by these antibodies have not been well-characterized, and their absolute specificity is not clear. A recent discovery that may assist in the purification of "potential stem cells" within the BMSC population is the finding that a monoclonal antibody (SB-10) recognizes the activated leukocyte-cell adhesion molecule (ALCAM) (Bruder *et al.*, 1998a). Because ALCAM is expressed on human BMSCs but is lost during developmental progression into differentiated tissues, SB-10 will likely be an important experimental tool for the study of osteoblast cell lineage. Through the use of new molecular biological technologies—such as subtractive hybridizations, differential display, SAGE (serial analysis of gene expression), and microarray technologies—it is hoped that new genes that are uniquely expressed by these cells will be identified. At the very least, should unique markers remain elusive, a pattern of gene expression, a so-called "fingerprint", will be generated through these types of analyses. With this information, development of reagents and screening strategies would allow for the identification of stem cells *in vivo* and the potential for cell sorting and further analysis *in vitro*.

A major gap in our knowledge relates to the fact that the true "stem-cell" character of BMSCs (*i.e.*, continuous self-renewal) has not been definitively proven in any animal species. If there is a true "stem-cell" present in this population, it will be necessary to determine the appropriate *ex vivo* culture conditions that will maintain their stem cell character (as has been done for embryonic stem cells used in the generation of transgenic animals), and then to induce them to differentiate into the desired phenotype through the modulation of their microenvironment, both *in vivo* and *in vitro*. Consequently, a better understanding of the hierarchy of CFU-Fs and BMSCs is needed. While it is now recognized that these popula-

tions display a variety of phenotypic expression patterns (osteoblastic, chondrogenic, hematopoiesis-supportive, adipocytic) both *in vivo* and *in vitro*, it is not understood how this phenotypic heterogeneity is established, maintained, and disrupted in disease states.

Animal studies have provided a great deal of encouragement; however, there are several obstacles that must be overcome prior to the use of BMSCs in human clinical protocols. First, large quantities of *ex vivo* expanded cells must be generated in an FDA-approved tissue culture facility in order to provide enough material for reconstruction of typically large skeletal segmental defects. True utility of BMSCs in skeletal regeneration will require major improvements in the current *ex vivo* expansion procedures to increase the yield and reduce the time required to generate sufficient numbers of cells. Given the inherent heterogeneity of the BMSC population, it may also be required to develop large-scale cloning procedures such that clones with desired phenotypic characteristics can be purified and used directly in tissue engineering protocols. For example, in the regeneration of craniofacial bones, which are primarily devoid of hematopoietic marrow, BMSCs clones that give rise only to bone without hematopoiesis would be more desirable than the use of multi-colony-derived strains that would generate bone along with hematopoietic stroma.

Second, there is a need for continued development of vehicles for the introduction and engraftment of these cells *in vivo*. Appropriate vehicles must be engineered and carefully selected depending on the site to be reconstructed (weight-bearing *vs.* non-weight-bearing sites, trabecular *vs.* cortical sites, etc.). It is likely that reconstruction of a particular skeletal site will require different vehicles, with or without bioactive factors, designed to direct the activity of BMSCs into specific expression patterns to regenerate bone in the desired shape and form.

Finally, if the use of BMSCs in gene therapy protocols is to be successful, better methods for stably or transiently transfecting normal human cells with high efficiency are critical. It can be envisioned that BMSCs can be either transiently or stably transfected to produce a protein that is missing or defective, and implanted within a patient to provide a missing or defective protein. However, expression of the desired protein product must be controlled such that the level of its production, post-translational modification, secretion, and localization to the desired active site is suitable. Selection of the appropriate promoter driving the ectopic production of the desired protein is of critical importance for physiologically significant expression and secretion and will require extensive knowledge of gene expression patterns on the part of the implant team.

While it is clear that there is much yet to be learned, it is also clear that the BMSC population provides a unique scientific opportunity for us not only to better our understanding of the processes of skeletal metabolism and homeostasis, but also to translate this information into clinical procedures that will provide a great deal of patient benefit.

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