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Marrow Stromal Stem Cells for Repairing the Skeleton

HERVÉ PETITE* AND DIDIER HANNOUCHE

Laboratoire de Recherches Orthopédiques, CNRS UPRES-A 7052, Université D. Diderot, Faculté de Médecine Lariboisière Saint-Louis, 10 avenue de Verdun, 75010 Paris, France

Introduction

A wound is a disruption of tissue integrity that is typically associated with a loss of substance. Its occurrence initiates a dynamic process of healing which aims at re-establishing tissue continuity. However, the degree of morphological and functional recovery that ensues will depend on the species, the organs and the amount of substance lost. For example, a bone fracture will heal through the formation of a soft callus that will gradually convert into a hard callus by endochondral ossification. Under favourable conditions, it will be remodelled and lead to a virtually complete restoration of the anatomical structure of the damaged bone. However, when a bone defect has reached a critical size, it heals through the formation of a non-functional scar tissue (Hollinger and Kleinschmidt, 1990). A key factor in the outcome of the healing process is the supply of a sufficient number of osteocompetent cells within the defect.

The notion that bone marrow is a potential source of osteocompetent cells is not new. As early as 1968, Friedenstein established that when bone marrow is plated at low cell densities, it gives rise to pluripotent fibroblastic colonies clonal in origin which, upon appropriate culture conditions, differentiate into osteoblasts, chondroblasts, adipocytes, and myelosupportive phenotypes (Friedenstein *et al.*, 1968). The cells, from which colonies originated, are fibroblastic in appearance and were initially termed colony-forming units-fibroblastic (CFU-F). Now, they are more often referred

*To whom correspondence may be addressed (hpetite@infobiogen.fr)

Abbreviations: CFU-F, colony-forming unit-fibroblastic; MSSC, marrow stromal stem cells; BMSF, bone marrow stromal fibroblast; BMP, bone morphogenetic protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor β; b-FGF, basic fibroblast growth factor; Dex, dexamethasone; AA2P, L-ascorbic acid 2-phosphate; MSSC-ALP+, MSSC positive for alkaline phosphatase; PGA, polyglycolic acid; Pla, polylactic acid; PLGA, co-polymer of PLA and PGA; O.I. osteogenesis imperfecta; CAT, chloramphenicol acetyl-transferase; OC, osteocalcin.

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to as either marrow stromal stem cells (MSSC) or mesenchymal stem cells. The progeny of these CFU-F are defined as bone marrow stromal fibroblasts (BMSFs). Only a subset of this population exhibits stem cell characteristics and is able to undergo extensive replication in culture. When placed in the closed environment of a diffusion chamber in reverse transplantation experiments, a proportion of these BMSFs will form bone and cartilage tissue (Friedenstein *et al.*, 1966, 1970, 1987; Ashton *et al.*, 1980; Thomson *et al.*, 1993). The vast majority of studies have been performed with the whole BMSF population with no further purification of the stem cell fraction as no clear-cut markers, which could lead to an efficient selection, are available. Consequently, in this review, we will use the acronym BMSFs which is, in the opinion of the authors, the most appropriate to reflect the heterogeneous nature of these cell populations. The possibility of expanding the number of BMSFs *in vitro* from a limited starting sample prompted their use for skeletal repair. Furthermore, BMSFs are easily transducible (Allay *et al.*, 1997; Marx *et al.*, 1999) and can also engraft into various tissues by means of systemic injection. This makes them interesting tools for the delivery of therapeutic genes and for the correction of genetic defects.

The recognition of these potentials has opened new avenues for repairing the skeleton. This review focuses on three areas: first, the use of BMSFs for the repair of large bone defects by local BMSF therapy; second, the use of BMSFs as a mean to deliver bone morphogenetic proteins (BMP), growth factors which have the potential to induce bone formation; and finally, the treatment of genetic skeletal disorders by systemic BMSF infusion. A special emphasis will be given to data pertaining to human BMSFs (hBMSFs).

Local BMSF therapy for the repair of bone defects

CLINICAL ISSUES

Bone healing is generally considered to be biologically optimal and the vast majority of defects will heal spontaneously with minimal treatment. However, among the 6 million fractures occurring every year in, for example, the United States, 5 to 10% will require further treatment for compromised healing because of interposition of soft tissue, improper fracture fixation, loss of bone, metabolic disturbances, impairment of blood supply or infection (Praemer *et al.*, 1992). In addition, there are specific clinical settings in which large pieces of bone must be resected to treat benign or malignant tumours or osteomyelitis.

In these challenging situations, autologous bone harvested from donor sites such as the iliac crest is the preferred treatment (Einhorn, 1995). Although it is the benchmark that all alternatives must meet or exceed, it has several limitations, including a limited supply of suitable bone and an additional morbidity to the donor site. Its harvest is painful and may be responsible for donor site fracture, herniation of abdominal content, infection, haemorrhage, cosmetic disability, and nerve damage. Alternatively, surgeons can use banked bone or synthetic or natural substitutes, but the success of these materials is limited as they only provide a scaffold, which needs to be invaded by bone-forming cells (Damien and Parsons, 1991; Shors, 1999). In fact, they will give good clinical results only if they are implanted in small defects and in direct apposition to bone.

With this in mind, a possible improvement of these materials could be achieved by loading them with bone-forming cells (Caplan, 1991; Goshima *et al.*, 1991; Bruder and Fox, 1999). This 'biological composite' will not depend on the local recruitment of osteocompetent cells for the synthesis of new bone, making it of particular interest for clinical cases in which the bed of the wound cannot provide these cells. This includes patients with large bone defects and those with a reduced number of osteocompetent cells because of ageing, osteoporosis, metabolic disturbances or irradiation.

CLINICAL SCENARIO

Figure 4.1 illustrates a likely scenario. *In vivo*, osteocompetent cells would produce osteoid along the wall of the pore of the biomaterial, whereas invading resorbing cells would erode progressively the biomaterial. A delicate balance between biomaterial resorption and bone formation should be achieved to allow the newly formed bone to become mechanically competent with the help, in the early phases, of an appropriate internal fixation.

SOURCES OF OSTEOCOMPETENT CELLS

There are a number of possible sources of cells for tissue engineering of bone, each with its own advantages and drawbacks. The osteogenic potential of fresh bone marrow has long been recognized and a number of investigators have experienced some success using autologous fresh bone marrow to augment bone formation in experimental studies (Ohgushi *et al.*, 1989; Grundel *et al.*, 1991; Okumura *et al.*, 1991; Wolff *et al.*, 1994). Connolly *et al.* (1991), Healey *et al.* (1990) and Garg *et al.* (1993) have documented the use of fresh bone marrow in the treatment of ununited fractures. However, no prospective randomized clinical trial has thus far been carried out to definitely demonstrate the efficacy of this practice. In addition, the quantity of newly-formed bone is directly dependent on the number of osteocompetent cells implanted, prompting the use of techniques allowing the amplification of the osteocompetent cell pool. Periosteal cells, osteoblasts and chondrocytes have also been evaluated (see Bruder and Fox (1999) for discussion). However, a consensus seems to emerge, at least for the repair of large bone defects, for the use of BMSFs, despite an interest in the simplicity of use of fresh bone marrow.

MARROW STROMAL STEM CELLS

Proliferative potential and senescence of BMSFs

A minimalist definition of stem cells is that 'they have the capacity both to self-renew and to generate differentiated progeny' (Morrison *et al.*, 1997). In others words, they should be able to make identical copies of themselves throughout the life of the organism and give rise to a mature progeny. Friedenstein had established that BMSFs arising from MSSCs can be maintained for up to 26 passages (Friedenstein, 1976). However, as all mammalian somatic cells, hBMSFs senesce after 38 doublings

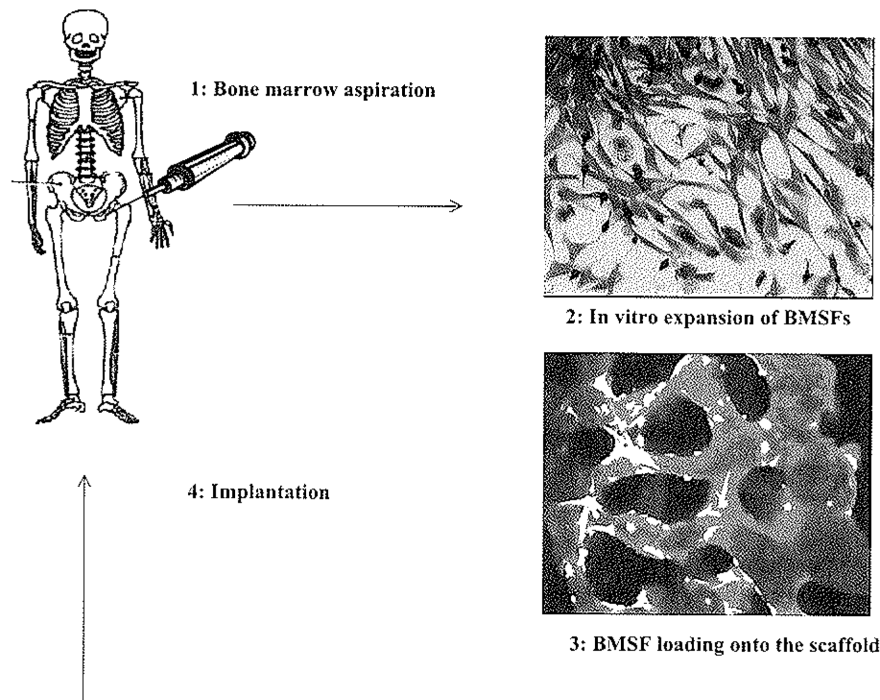

Figure 4.1. A clinical scenario for BMSF local cell therapy.

(Bruder *et al.*, 1997). Because of this, they should rather be considered as a subset of stem cells, which has a limited capacity to renew. In addition, there are no published data concerning the possibility of subtle changes that might occur when BMSFs are expanded. In other words, the question as to whether aged BMSFs are implanted remains open and of importance. Recently, a study suggested that senescent fibroblasts were able to promote premalignant and malignant, but not normal, epithelial cell growth (Krtolica *et al.*, 2001). From a tissue engineering point of view, the number of BMSFs that could be harvested from a bone marrow aspiration seems sufficient. Thus, Friedenstein determined that kilograms of bone could be generated from the pelvic marrow of a single rabbit (Friedenstein *et al.*, 1987). In addition, a number of studies have shown that hBMSFs have high proliferative potential (Haynesworth *et al.*, 1992; Bruder *et al.*, 1997; Jaiswal *et al.*, 1997) and calculated that almost half a billion cells could be obtained at passage 6 by starting with 100 to 500 adherent MSSCs (Haynesworth *et al.*, 1992). These results have obvious implications as a large number of osteocompetent cells can be obtained from a small marrow aspiration.

In vitro *expansion of hBMSFs*

In humans, the adherent layer of BMSFs is rather homogeneous, consisting of elongated fibroblastic cells with a small number of macrophages that decreases with consecutive passages (Bentley and Foidart, 1980; Golde *et al.*, 1980; Wilson *et al.*,

1981). Expansion of hBMSFs is carried out in calf serum, which provides key factors for cell attachment, proliferation and differentiation. However, a number of disadvantages pertain to the use of calf serum in a clinical setting, including batch-to-batch variations which render serum screening mandatory to obtain consistent hBMSF amplification (Lennon *et al.*, 1995). A serum-free, chemically-defined medium has been established for culturing the STRO-1 fraction of hBMSFs and comprises α-MEM, supplemented with 10 μg/ml bovine-derived pancreas insulin, 2% bovine serum albumin, 80 μg/ml human, low density lipoprotein, 200 μg/ml iron-saturated human transferrin, 2 mM L-glutamine, 10^{-8} dexamethasone (Dex), 100 μM L-ascorbic acid-2-phosphate and 5×10^{-5} M β-mercaptoethanol (Gronthos and Simmons, 1995). In these conditions, EGF and PDGF and a pre-coating of the dish by fibronectin are required to promote growth of hBMSF. In slightly different conditions, the use of neutralizing antibodies against PDGF, TGF-β, bFGF and EGF in serum-free medium conditioned by marrow cells specifically suppressed BMSF colony formation, with the most profound inhibition obtained with anti-PDGF and anti-TGF antibodies (Kuznetsov *et al.*, 1997a). Unfortunately, currently used techniques carry risks of transmission of zoonoses through the use of animal-derived products. This could be a problem if those cells are to be implanted back to patients. They should, therefore, be used only as a second choice, the first choice being the culture of hBMSFs in the patient's own serum. Cultures of cartilage cells have been performed with success in these conditions.

Dex is used as a standard supplement for the *ex vivo* amplification of hBMSFs, as a number of studies suggest that it enhances the recruitment and further differentiation of osteoprogenitors from the heterogeneous population of hBMSFs *in vitro* (Beresford *et al.*, 1994; Cheng *et al.*, 1994; Jaiswal *et al.*, 1997; Walsh *et al.*, 2001). Dex is a glucocorticoid with complex effects on hBMSFs, dependent on the extent of cellular differentiation and the species of origin. Its effects vary according to the dose and time of exposure. In a cohort of 30 patients, Walsh *et al.* (2001) observed that, at supra-physiological concentrations (100 nM and above), Dex consistently decreases cell proliferation, whereas at physiological concentration (10 nM), it has minimal effect on cell proliferation (Walsh *et al.*, 2001). At concentrations varying from 100 pM to 1 μM, Dex increases the alkaline phosphatase expression in a dose-dependent manner. Both the number of cells and the ALP activity per cell are increased. Of importance is the fact that effects of Dex are donor-dependent. On individual analysis, Walsh observed that at 10 nM, Dex decreased, increased, or had no effect on cell number, depending on the donor. Furthermore, marked differences in the increase in ALP activity have been observed among patients (Cheng *et al.*, 1994; Jaiswal *et al.*, 1997; Walsh *et al.*, 2001), prompting further investigations to optimize Dex concentrations on an individual level. The positive influence of Dex on the expression of the osteogenic potential of hBMSFs is also suggested by *in vivo* studies. Thus, when hBMSFs are expanded in the presence of 10 nM Dex and transplanted intraperitoneally in a closed system such as diffusion chambers, they will form bone. hBMSFs cultured in the absence of Dex will only form a fibrous tissue (Haynesworth *et al.*, 1992; Gundle *et al.*, 1995). It has been proposed that Dex is necessary for bone formation when hBMSFs are poorly osteogenic, or when hBMSFs are loaded into vehicles that poorly support osteogenesis, such as gelatin capsules (Krebsbach *et al.*, 1997; Kuznetsov *et al.*, 1997b).

The osteogenic potential of hBMSFs is maximized by the addition of β-glycerophosphate (β-gly) and ascorbic acid. Ascorbic acid acts as a co-factor in the hydroxylation of proline and lysine residues in collagen, and increases procollagen translation (Schwarz, 1985). There has been a proposal to replace it with the long-acting ascorbate analogue, L-ascorbic acid-2-phosphate (AA2P). AA2P has a half-life of 7 days and its use avoids the tedious daily addition of ascorbic acid under the conditions that prevail in culture (Hata and Senoo, 1989). Addition of AA2P to hBMSFs cultured in DMEM in the presence of Dex and β-GLY increases both cell proliferation and ALP activity at day 8 (Jaiswal *et al.*, 1997). Addition of FGF-2 has also been proposed as FGF-2-expanded BMSFs have been shown to yield more bone formation when loaded onto ceramics and implanted *in vivo* (Martin *et al.*, 1997). In a clinical situation, the benefits of the addition of biological compounds must outweigh the possible sanitary risks. Of importance is the fact that growth factor requirements and response might be different from patient to patient and be influenced by age. For example, an increase in responsiveness to exogenous TGF-β in mice BMSFs is observed in older animals, whereas this increase is either not seen, or seen at lower levels, in young animals (Gazit *et al.*, 1999). Additionally, bone cells are sensitive to mechanical stresses. A safe and elegant way to modulate their proliferation and differentiation might include growing them under fluid flow.

Age

Bone regenerative therapy would be of major interest in the elderly patient. The question as to whether the number of MSSCs residing in bone marrow declines with age is therefore critical. One approach to answer this question is to use an *in vitro* colony assay. The number of colonies reflects the number of stem cells. Its size is indicative of the proliferation potential of the stem cell, whereas the number of colonies expressing alkaline phosphatase, a bone marker, represents the number of osteoprogenitor cells (MSSC-ALP+). Studies seeking to determine the influence of age on the prevalence of MSSCs in humans have yielded conflicting results until recently. Results have often been obtained from a limited number of donors suffering from various diseases, and the extent to which they are truly representative is therefore uncertain. Different sites and techniques of bone marrow sampling, as well as a variety of protocols of MSSC isolation, have biased the results. On the basis of recent studies, a consensus seems to emerge on the evolution of the number of MSSC during a lifetime. Rather than a linear age-related decline in MSSC-ALP+ over a lifetime, there is a sharp decline in MSSC-ALP+ when skeleton dynamics switches from a growth pattern to a maintenance pattern. This hypothesis is in accordance with D'Ippolito and co-workers (D'Ippolito *et al.*, 1999), who have observed that the number of MSSC-ALP+ obtained from vertebral bodies of healthy donors aged 3–36 ($66 \pm 9.6/10^6$ cells) was significantly higher than the number of MSSC-ALP+ of individuals aged 41–70 ($14.7 \pm 2.6/10^6$ cells). The authors failed to note any further decline in the osteoprogenitor pool after age 40. This hypothesis is also consistent with data by Nishida and co-workers that showed a much higher number of MSSC-ALP+ in children under the age of 10 ($52.4/4 \times 10^6$ cells) when compared to individuals ranging from 10 to 19 ($17 \pm 5/4 \times 10^6$ cells) and a gradual but limited decrease in MSSC-ALP+ thereafter (Nishida *et al.*, 1999). Majors and co-workers

have also found an age-related decline in MSSC-ALP+ in bone marrow harvested from the iliac crest, but did not look for a 'biphasic decline' (Majors *et al.*, 1997). In a cohort of 57 individuals subjected to an elective orthopaedic procedure, Muschler and co-workers have failed to observe any decline in MSSC-ALP+ prevalence with age (Muschler *et al.*, 2001). However, it should be noted that, in that study, the mean age was approximately 60 years old, with only 7 individuals younger than 30. Finally, Stenderup and co-workers did not find any age-related decline in the number of MSSC-ALP+ in a cohort of individuals ranging from 22 to 83 years old (Stenderup *et al.*, 2001). Taken together, all these studies suggest that, in humans, there is a sharp decline in MSSC-ALP+ prevalence when skeletal growth is achieved, rather than a linear decrease with age.

From a cell therapy point of view, the isolation of BMSFs was almost always possible in these studies; although further investigations are needed to assess the possibility of consistently expanding these cells.

Studies have found that the proliferative potential of BMSFs is either unchanged or diminished in elderly patients (Oreffo *et al.*, 1998; D'Ippolito *et al.*, 1999; Stenderup *et al.*, 2001). Further studies will be required to provide a better understanding of the influence of ageing on the BMSF proliferative potential.

Site of harvest

Marrow is localized in the cavities of the long bones and the interstices of the spongiosa in vertebral bodies, ribs, sternum and the flat bones of the cranium and pelvis. A shift from red, haematopoietically-active marrow to a relatively inactive fatty marrow occurs with age, leaving adult red marrow only in the proximal ends of the humerus, in vertebrae, ribs, sternum and iliac crest. BMSFs can be obtained from these tissues but, to the knowledge of the authors, there is no study quantifying their respective osteogenicity. This would be of interest, as studies suggest a common precursor to osteoblasts and bone marrow adipocytes (Bennett *et al.*, 1991; Park *et al.*, 1999) and there is an inverse relationship between the differentiation of adipocytic and osteogenic cells (Beresford *et al.*, 1992). Although no clinical data exist on the influence of the site of sampling on the osteogenicity of bone marrow in a healthy population, the number of MSSC in tibial marrow of patients with rheumatoid arthritis (8.2 ± 5.6) is significantly lower than the number of MSSC in iliac marrow (28 ± 9.8) (Suzuki *et al.*, 2001). Further investigations are needed to determine if these differences are due to the inflammatory response occurring nearby joints affected by rheumatoid arthritis, or are due to differences of the osteogenicity of bone marrow.

More recently, skeletal stem cells, which share with MSSCs the ability to generate at least three phenotypes (osteoblasts, adipocytes, and reticular cells) and are able to form bone *in vivo*, were isolated from whole blood in mouse, rabbit, guinea pig and humans (Kuznetsov *et al.*, 2001). Similarly, Zvaifler and co-workers have isolated $CD34^-$ mononuclear cells with *in vitro* characteristics that fulfilled criteria for MSSCs from blood (Zvaifler *et al.*, 2000). These results are in conflict with Lazarus and co-workers, who failed to isolate MSSC from patients who had undergone chemotherapy or cytokine therapy prior to collection (Lazarus *et al.*, 1997). Taken together, these results raise the possibility of isolating MSSCs from blood in healthy

patients, although further studies are needed to clarify the influence of chemotherapy or cytokine therapy on the mobilization of skeletal stem cells.

THE DESIGN OF THE DELIVERY VEHICLE

Whereas a consensus exists on the use of bone marrow as the preferred source of osteocompetent cells, selecting the most suitable scaffold to deliver the cells is still the subject of debate. A plethora of biodegradable materials has been proposed over the past 30 years, but the data available on the ideal physico-chemical characteristics that they should exhibit are varied. None of these has yet satisfied all the requirements for tissue regeneration. More than a simple delivery vehicle allowing the transplantation of large populations of cells, the scaffold must promote cell proliferation and differentiation and should encourage angiogenesis. When implanted *in vivo*, neither the scaffold, nor its degradation products, should induce any adverse reaction and, ideally, it should degrade at a predefined rate that matches the rate of new bone formation. Among all the properties that the scaffold should meet to provide a suitable environment for tissue repair, the architecture of the template appears to be crucial. Of paramount importance are the porosity and the permeability of the scaffold: the porosity provides space for cells, blood vessels, and the newly-formed extra-cellular matrix, and permeability allows nutrients to flow through the template, as well as the elution of metabolic waste (Shors, 1999; Hutmacher, 2000; Agrawal and Ray, 2001). Pore sizes greater than 150 μm encourage the ingrowth of mineralized bone.

To date, two groups of scaffolds have been extensively evaluated in the field of bone tissue engineering, namely calcium phosphate ceramics (CPC: hydroxyapatite, tricalcium phosphate, etc.), and synthetic polymers (Damien and Parsons, 1991; Shors, 1999; Hutmacher, 2000). CPC are attractive because of the similarity that exists between their formula and that of the mineral phase of bone. However, they are brittle and very difficult to shape. Synthetic polymers are biocompatible, biodegradable, and can be designed in the form of sponges, open lattices with interconnected fibres and, most recently, composites in specific shapes. A variety of polymers exist, the most widely studied being polyglycolic acid (PGA), polylactic acid (PLA) and the co-polymer of PGA/PLA (PLGA) (Freed *et al.*, 1994; Athanasiou *et al.*, 1996; Hutmacher, 2000; Agrawal and Ray, 2001). These polymers are amenable to chemical manipulation for improving cell adhesion and controlling the degradation rate based on the ratio of lactide to glycolide. They have been studied extensively for different applications in tissue engineering (bone, cartilage, blood vessel, nerve conduits, liver) and provide many advantages compared to other scaffolds. They can be manufactured in three-dimensional structures that would fit the patient's defect perfectly. As different polymers, each supporting a specific tissue, have been developed (Martin *et al.*, 2001), it can now be envisaged to suture them together for the repair of osteochondral defects. Finally, they can be combined with growth regulatory molecules that would enhance the proliferation and differentiation of the implanted cells (Richardson *et al.*, 2001). However, while experimental results with these polymers have been promising, their surgical application in clinical practice is still limited. Their degradation *in vivo* produces pockets of acidic monomers that may alter cell proliferation and function and unleash an inflammatory reaction (Athanasiou *et al.*, 1996; Hutmacher, 2000; Ignatius *et al.*, 2001). Furthermore, these

polymers are hydrophobic and their surface is less than optimal for cell attachment.

Overall, a number of biodegradable scaffolds have been proposed over the past decade, some being commercialized, and others in pre-clinical testing. Even though real progress has been made in this field, several challenges lie ahead before moving to the clinical application, and the search for the ideal material is still ongoing.

Experimental studies

The osteogenic potential of BMSFs *in vivo* was initially observed in studies in which the cells were placed into diffusion chambers or under the renal capsules. In orthopaedics, Goshima and co-workers have raised the possibility of using these cells in combination with ceramics to accelerate bone formation (Goshima *et al.*, 1991). Goshima *et al.* showed that cultured BMSFs loaded into ceramics and implanted subcutaneously in rats induced bone formation within two weeks after implantation, whereas supplementation of ceramic templates with skin or muscle fibroblasts did not have any positive influence on bone formation. Osteogenesis appeared to be dependent on the density of BMSFs suspension (Goshima *et al.*, 1991), as this was described previously with the implantation of BMSFs in diffusion chambers (Mardon *et al.*, 1987). Depending on the species, the amount of bone formation was correlated or not to the number of cell passages before implantation. While, in rats, osteogenesis was lower with the increase of cell passages, no relation was observed in humans when BMSFs were evaluated in the ceramic cube assay, as long as the cells retained their proliferative status (Haynesworth *et al.*, 1992; Krebsbach *et al.*, 1997). Subculturing rat BMSFs in the presence of dexamethasone increased their osteogenic potential in a similar subcutaneous model (Yoshikawa *et al.*, 1996). This was not found to be true with hBMSFs, providing that cells were loaded on ceramic scaffolds (Krebsbach *et al.*, 1997). Interestingly, frozen storage did not affect this potential with rat or human BMSFs (Goshima *et al.*, 1991; Bruder *et al.*, 1997).

The primary clinical indication of osteogenic materials is the repair of large bone defects. Therefore, studies have assessed the efficiency of the repair of critical-sized femoral bone defects in rat. The bone-regenerating ability of osteogenic implants was always greater than the bone-regenerating ability of either the scaffold alone or the defects left empty (Kadiyala *et al.*, 1997; Bruder *et al.*, 1998b).

However, in this model, the size of the defect was limited (6 mm). To show clinical applicability of tissue-regeneration therapies, the ability of osteogenic materials to repair defects of clinically relevant volumes was evaluated in load-bearing situations in large animals, using as a scaffold either a synthetic hydroxyapatite-tricalcium phosphate ceramic (Bruder *et al.*, 1998a), or a porous hydroxyapatite ceramic (Kon *et al.*, 2000). Experimental conditions are summarized in *Table 4.1.* These studies showed an increase in osteogenesis in BMSF-loaded ceramics when compared to the use of a scaffold alone. However, no resorption of the scaffolds has been observed within the time-frame of the experiments with hydroxyapatite-tricalcium phosphate or the hydroxyapatite ceramic.

Another study was carried out at the author's institution to explore the possibility of using natural coral exoskeleton as a cell delivery system (Petite *et al.*, 2000). Natural coral combines good mechanical properties with a rapid rate of resorption and an open porosity, making it an interesting candidate for the repair of large skeletal

Table 4.1. Evaluation of scaffolds for MSC in large animal models.

	Bruder *et al.* (1998)	Kon *et al.* (2000)	Petite *et al.* (2000)
Size of the defect (mm)	21	35	25
Osteosynthesis	internal plate	external fixator	internal plate
Species	Dog	Sheep	Sheep
Number of MSC (10^7 cells)	3.75	5 to 10	3.25
Scaffold characteristic			
composition	65% HAP 45% β-TCP	100% HAP	99% calcium carbonate
Mean pore size (μm)	200–450	14% < 150/86% > 150	150–400
Size (mm × mm)	21 × 14	–	25 × 14
Coating	human plasma fibronectin	fibrinogen*	no
Follow-up	16 weeks	8 weeks	16 weeks

* Implants have been soaked in cells suspended in a fibrinogen solution, which is polymerized thereafter with thrombin.

defects predominantly subjected to compressive loads. The bone hybrid, consisting of calcium carbonate, was assessed in a large segmental defect model in sheep. The tissue-engineered bone elicited more bone formation than that obtained with the scaffold alone. Corticalization and the formation of a medullary canal with mature lamellar bone were observed in the most favourable cases (*Figure 4.2*). Unfortunately, clinical unions were obtained in only 3/7 operated limbs. These results are encouraging; however, further studies are needed to demonstrate 100% successful results in animals prior to moving to clinical trials.

CLINICAL DATA

To the authors' knowledge, there are only two reported studies on the use of osteoprogenitor cells for the repair of bone defects in humans. Both are short case reports, on one and three patients, respectively.

In the first report, Quarto and co-workers displayed promising results with the use of hBMSFs loaded on hydroxyapatite scaffolds to treat large bone defects (Quarto *et al.*, 2001). There were three mid-diaphyseal bone losses, ranging from 4–7 cm, in patients aged 16 to 41 years old. Bone healing was achieved in 2 months, with good integration at the interfaces with the host bone, and without any adverse reaction. However, owing to the nature of the article itself, which was a short correspondence, it is hard to conclude whether the technology applied here was a clinical success and did improve the outcome of the patients compared to a standard bone autografting. No mention was made of the previous treatments applied, neither on the length nor the conditions of cell culture. Furthermore, careful examination of provided radiographs 8 to 18 months after surgery did not show any biodegradation of the scaffold compared to the postoperative X-rays. Resorption rates of calcium phosphate ceramics vary inversely with the calcium phosphate ratio, and hydroxyapatite is known to be almost undegradable when implanted *in vivo*. It is likely that the choice of the scaffold here was non-optimal.

More recently, Vacanti and co-workers have reported the case of a 36-year-old man who had a traumatic avulsion of the distal phalanx of the thumb (Vacanti *et al.*, 2001). Reconstruction of this bone was performed using culture-expanded osteoprogenitor

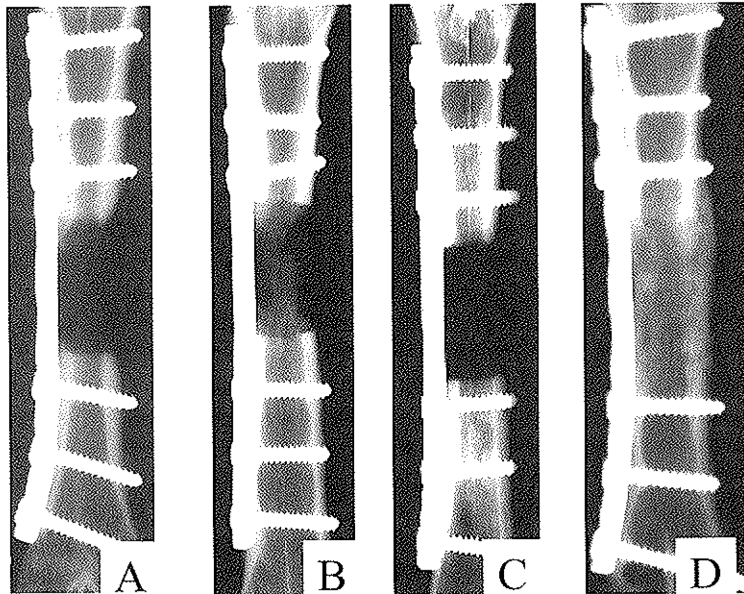

Figure 4.2. X-rays of the sheep metatarsus either left empty (A) or filled with coral alone (B), coral and fresh bone marrow (C), or coral and BMSFs (D) taken 16 weeks after a 25 mm resection. Note the absence of bone formation within the defect in A, B and C. In D, bone formation was sufficient for union of the defect after 16 weeks.

cells isolated from the periosteum and seeded on a pre-shaped coralline scaffold. At 28 months' follow-up, the thumb had normal length, but strength was reduced compared to the opposite side (25%), and no active motion was observed at the interphalangeal joint. A biopsy of the implant performed 10 months after surgery showed only 5% of newly formed bone within the pores.

Although non-optimal, these studies definitely show that the use of local cell therapy for the treatment for bone defects is feasible, and these modalities will probably supersede the need for bone grafting in the near future. However, to clearly demonstrate the benefits and the superiority of these expensive technologies, they should be objectively compared to established treatments in prospective randomized trials.

Use of BMSFs for the delivery of BMP

Bone healing involves migration, proliferation and differentiation of undifferentiated cells under the concerted control of the surrounding extracellular matrix, soluble effectors such as growth factors, and the mechanical milieu. It has been hypothesized that altering levels of specific growth factors such as BMP will encourage key events of the bone repair cascade, as these growth factors have the unique characteristic to induce undifferentiated cells into an osteogenic pathway. Although encouraging results have been obtained by exogenous addition of recombinant BMP loaded within

a collagen scaffold (Li and Wozney, 2001), the challenge still remains of being able to deliver these molecules with the appropriate kinetics. A major discrepancy exists between the time-course of bone healing, which occurs over a period of weeks, and the short half-life of growth factors, which ranges in the order of minutes to hours. A practical consequence of this difficulty is illustrated by the high dosage of BMP necessary to induce bone in clinically relevant defects (Kirker-Head *et al.*, 1995). An alternative to the delivery of BMP at the protein level would be to introduce the gene encoding BMP into BMSFs. The procedure is termed an *ex vivo* gene therapy (Evans and Robbins, 1995; Winn *et al.*, 2000). Ideally, the transduced cells loaded into a scaffold and placed within the defect would then act as a 'mini-factory', producing a sustained release of the growth factor in a biologically active form for many weeks. This therapeutic strategy brought bone healing one step further, as the fracture site will be supplied with three key factors for bone formation: osteocompetent cells, osteoinductive growth factors, and a scaffold that provides a surface for bone deposition. The clinical scenario will include the harvest of BMSFs, their transfection by the therapeutical gene, and their reimplantation in the same patient in combination with a scaffold.

Lieberman *et al.* (1999) showed that rat BMSCs transduced by means of a BMP-2 expressing E1 defective adenoviral vector were able to heal an 8 mm defect in rats. Out of the 24 defects that received transduced cells, 22 healed by the formation of a coarse trabecular bone. The two defects that failed to heal in the BMP-2 group revealed a decrease in the transgene expression as measured *in vitro*, stressing the need for specific and individual control of transgene expression to ensure a 100% success. However, if these two failures were eliminated from the biomechanical analysis, there was no significant difference in energy to failure or torque to failure between the defects treated with BMP-2-transduced cells and intact femora, suggesting that the BMP-2-induced bone was mechanically similar to native bone. Although adenoviral proteins are immunogenic and known in similar systems to decrease (Musgrave *et al.*, 1999) or suppress BMP expression (Okubo *et al.*, 2000), the authors did not report any adverse reactions in their study. Retroviral transduction of BMSFs has also been achieved successfully (Allay *et al.*, 1997; Marx *et al.*, 1999). Retroviruses may not be suitable vehicles for BMP for three reasons: 1) transgenes inserted by means of retroviruses are inserted within the genome, carrying the risks of insertional mutations; 2) gene expression might extend over a too long time period; and 3) retroviruses infect only dividing cells (Evans and Robbins, 1995; Winn *et al.*, 2000). In this regard, insertion of genes by means of adenoviral vectors seems more promising as the adenoviral genome remains episomal, and therefore the expression of the transgene is transient, and a high transfection rate is observed as they infect non-dividing cells. However, new generations of less immunogenic adenoviruses are critically needed before going to clinical trials. In fact, most studies have been performed with immunocomprised animals.

Gene therapy for the healing of bone is in its infancy and a number of different approaches are being explored, including direct gene transfer, gene activated matrix, or *ex vivo* gene transfer (Evans and Robbins, 1995; Winn *et al.*, 2000). Most studies stressed the importance of a better control on the level of expression of the transgene. Overexpression of BMP has been shown to be toxic to cells *in vitro* (Mason *et al.*,

1998) and could lead to the formation of benign bone tumours *in vivo*. In addition, transduced cells should be maintained within the defect. Their systemic dispersion could induce inappropriate ectopic bone formation (Sale and Storb, 1983). In this regard, the use of tissue-specific promoters, which could exert control on the localization of the transgene expression, might be of great interest.

Systemic BMSF therapy

The potential of BMSFs to engraft into multiple tissues upon systemic transplantation has stimulated interest in their use as a means of correcting hereditary disorders, such as osteogenesis imperfecta, via systemic infusion of BMSFs.

When BMSFs from normal mice are infused via the circulation to irradiated transgenic mice with a phenotype of osteogenesis imperfecta (O.I), a small number of cells of donor origin are detected in a number of non-haematopoietic tissues via a PCR assay (Pereira *et al.*, 1998). In addition, primary cultures of fibroblastic cells of donor origin were established from a number of organs, including cartilage, calvaria and long bones, confirming the survival after engraftment of BMSFs (Pereira *et al.*, 1998). In the foetal lamb model, hBMSFs, transplanted in the foetal peritoneal cavity, could be found in pre-natal and post-natal haematopoietic and lymphopoietic sites, as well as multiple non-haematopoietic tissues, including adipose tissue, lung, articular cartilage, or cardiac and skeletal muscle, albeit at low frequency (Liechty *et al.*, 2000). Taken together, these studies demonstrate that BMSFs, systemically infused, can engraft in multiple tissues.

Evidence that functional bone cells could arise from transplanted cells was found by Nilsson and co-workers (Nilsson *et al.*, 1999). These authors observed that, after infusion of whole marrow, active osteoblasts and osteocytes of donor origin could be detected by *in situ* hybridization in femur sections of non-ablated mice for up to 6 months after transplantation (Nilsson *et al.*, 1999). More relevant to BMSFs, Hou and co-workers have transfected mouse BMSFs with a reporter gene (chloramphenicol acetyltransferase: CAT) under the control of osteocalcin (OC) promoter and infused them intravenously into recipient mice (Hou *et al.*, 1999). As expression of the reporter gene (OC-CAT) is restricted to bone tissue, the detection of OC-CAT activity in osteoblasts and osteocytes provided evidence that functional bone cells could arise from transplanted stroma-related preparation.

These data raise the possibility of using systemic infusion of BMSFs to cure genetic diseases such as O.I, an autosomal dominant negative disease in which one of the two genes encoding for type I collagen is mutated. Bone deformity, excessive bone fragility, and short stature are generally observed in this pathology. As it is a dominant negative disease, it appears as a good candidate for a cell therapy approach. A clinical trial has recently been conducted to treat children suffering from severe O.I (Horwitz *et al.*, 1999). In this pilot study, Horwitz and co-workers have demonstrated a successful engraftment of allogenic BMSFs injected intravenously in three children. The conditioning regimens received by the patients included a chemoablation and a total body irradiation in one case. Despite the low level of osteoblast engraftment (1.5–2% donor cells), the authors reported spectacular therapeutic effects following the bone marrow transplantation: improvement in bone histology, increases in total body mineral content, and a reduced rate of fractures. However, all these measure-

ments can be confounding, as they are hard to perform and to interpret, and further careful studies need to be achieved before pursuing these attempts, particularly considering the potential hazardous nature of the procedure (Bishop, 1999; Gerson, 1999; Marini, 1999; Bianco and Robey, 2000). Further investigations in experimental models are critically needed to determine the adequate dose of BMSFs, to improve the level of osteoblast engraftment, to assess their long-term persistence, as well as to evaluate the level of substitution to the recipient bone cells.

Conclusions

The use of BMSFs via systemic infusion for correcting genetic defects, albeit seductive, still requires substantially more research before moving to clinical trials. This includes an increase in efficiency of engraftment and a demonstration of biological efficacy of the infused BMSFs. In contrast, fruitful strategies based on the local transplantation of BMSFs loaded on a scaffold has led to effective repair of critical-size defects in healthy animals. Whereas preliminary clinical studies have shown that this strategy could be an interesting alternative for the treatment of large bone defects, further prospective randomized trials are needed to compare it objectively to established treatment. Additionally, most experimental models do not mimic the conditions that are often encountered clinically, including compromised vascularity and extensive scar tissue, prompting further assessments of this technique in clinically relevant experimental models to better assess its clinical indications.

The improvement in repair capacity brought by stem cells in mammals, although impressive, is yet limited when compared to the repair capacity of amphibian urodeles, such as salamanders. These animals are able to regenerate complex structure, including the limbs, upon amputation through the local formation of a mesenchymal growth zone. This potential relies on the ability of differentiated cells to re-enter the cell cycle, while maintaining positional identity. Although transfer of these capacities to mammals remains elusive, it cannot be discounted. Recent advances have given us a convenient source of stem cells. A better understanding of the molecular clues underlying positional identities is now called for.

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