The use of adult stem cells in rebuilding the human face

Pamela Gehron Robey, PhD; Paolo Bianco, MD

Judging from the explosion of articles not only in scientific journals, but also in the mass media and on the Internet, one could say the term "stem cells" has become linked to the word "cure."[1] Patients are becoming increasingly aware that stem cells may benefit them in treating whatever disorder they or their loved ones have. This includes orofacial problems, which have a high impact not only on facial appearance, but also on quality of life—specifically on the ability to chew, a function that is easily taken for granted until lost.

Despite all the enthusiasm about the discovery of stem cells and their great potential, there also is no doubt that in many cases, the applications of and the cures related to stem cells are not just around the corner, as the media would lead one to think. However, a great deal of progress has been made in a relatively short time.

Stem cells are not science fiction, but something that one day will become a part of each dentist's clinical practice. The immediate challenge is for dentists not only to be better able to address the questions that their patients have concerning stem cell–based therapy, but also to familiarize themselves with the

ABSTRACT

Background. Stem cells have been isolated from a variety of embryonic and postnatal (adult) tissues, including bone marrow. Bone marrow stromal cells (BMSCs), which are non–blood-forming cells in marrow, contain a subset of skeletal stem cells (SSCs) that are able to regenerate all types of skeletal tissue: bone, cartilage, blood-supportive stromal cells and marrow fat cells.

Methods. Bone marrow suspensions are placed into culture for analysis of their biological character and for expansion of their number. The resulting populations of cells are used in a variety of assays to establish the existence of an adult SSC, and the ability of BMSC populations to regenerate hard tissues in the craniofacial region, in conjunction with appropriate scaffolds.

Results. Single-cell analysis established the existence of a true adult SSC in bone marrow. Populations of ex vivo expanded BMSCs (a subset of which are SSCs) are able to regenerate a bone/marrow organ. In conjunction with appropriate scaffolds, these cells can be used to regenerate bone in a variety of applications.

Conclusions. BMSCs have the potential to re-create tissues of the craniofacial region to restore normal structure and function in reconstructing the hard tissues of a face. Ex vivo expanded BMSCs with scaffolds have been used in a limited number of patients to date, but likely will be used more extensively in the near future.

Key Words. Adult stem cells; skeletal stem cells; bone regeneration; tissue engineering; biomaterials.

JADA 2006;137:961-72.

Dr. Robey is the chief, Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Department of Health and Human Services, 30 Convent Drive, MSC 4320, Bethesda, Md. 20892, e-mail "probey@dir.nidcr.nih.gov". Address reprint requests to Dr. Robey.

Dr. Bianco is a professor of pathology, Department of Experimental Medicine and Pathology, Universita degli Studi di Roma "La Sapienza," Rome, and vice director and unit head, Institute of Cell Biology and Tissue Engineering, Parco Scientifico Biomedico San Raffaele, Rome.

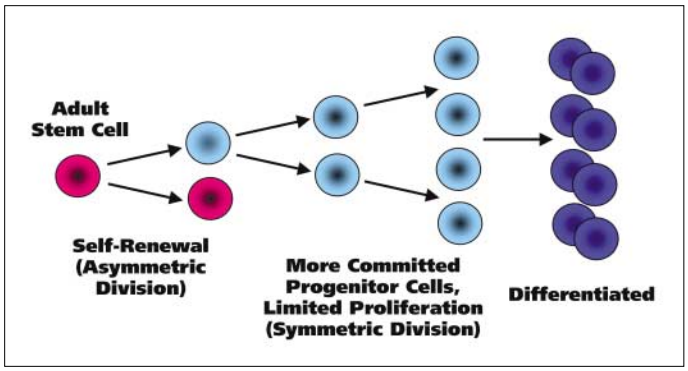

Figure 1. An adult stem cell (red) is defined by several properties, including its ability to "self-renew"—that is, when the stem cell is stimulated to divide, it gives rise to one daughter cell that is more committed to forming a particular cell type (a progenitor) (light blue) and one daughter cell that remains a stem cell (asymmetric division). Committed progenitors are thought to have a limited capacity for proliferation by symmetric division; their daughter cells then form differentiated cells within the tissue.

spectrum of tools they may have in the near future to restore form and function effectively.

This article reviews what we know and do not know about different types of stem cells—what is real and what may not be real with respect to their biological activity—and it offers a description of how stem cells can be used for the restoration of the bones of the human face.

WHAT IS A STEM CELL?

While listening to a conversation among a group of five people of different educational and scientific backgrounds, one might decipher at least 10 definitions of a stem cell. Much of the confusion lies in the fact that there are different types of stem cells, along with different expectations of their biological activity.[2] However, in keeping one's eye toward the future of stem cell biology in dental practice, it is important to understand the terminology that is applied to the various types of stem cells and how these terms relate to their different properties.

Definitions. There are three defining features of a stem cell on which all can agree.[3]

- A stem cell "self-renews"—that is, when a stem cell is called into action, it undergoes cell division. One daughter cell remains a stem cell, while the other becomes more committed to forming a particular cell type (a "committed progenitor") by a process called "asymmetric division" (Figure 1).
- A stem cell forms multiple cell types (that is, it is "multipotent").
- A single stem cell completely re-forms a particular tissue when it is transplanted within the body.

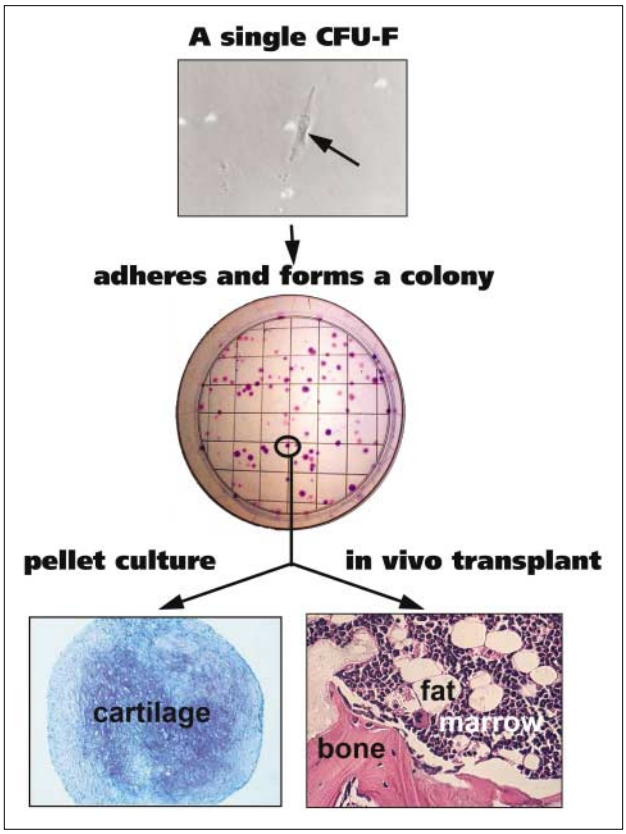

Figure 2. Proof of the existence of a multipotent skeletal stem cell in adult bone marrow. When single-cell suspensions of bone marrow are plated at low density, a single, adherent colony-forming unit–fibroblast (CFU-F) attaches to the substrate and proliferates to form a colony. When individual colonies are analyzed, 10 to 20 percent of them are able to form cartilage when grown in a high-mass pellet culture. When they are transplanted in conjunction with appropriate scaffolds in vivo, they are able to form bone, marrow fat and the stroma that supports blood formation and, therefore, are multipotent.

On the basis of these three defining features, several others are implied, but are not necessarily true of all stem cells[4]:

- *Self-renewal = extensive proliferation:* The ability to self-renew has been linked conceptually to a stem cell's ability to divide extensively to form vast numbers of cells. However, a stem cell is not immortal, but is endowed with a certain restricted capacity to self-renew related to how fast a tissue turns over.
- *Clonogenicity = stemness:* A stem cell is thought to be "clonogenic," which means that it can proliferate to form a colony of cells (Figure 2). However, while clonogenicity is part of the essential assay in defining a stem cell (that is, a single cell capable of proliferating and forming multiple cell types), not all cells that form colonies qualify as stem cells.
- *Stemness = undifferentiation:* In many cases, a

stem cell is thought to be an undifferentiated cell type (that is, it does not have a mature phenotype), but there are instances in which a cell with differentiated character can behave as a stem cell.

Types of stem cells. Although all stem cells share the three characteristics listed above, they are not necessarily equal in their ability to form multiple cell types, and a hierarchy exists:[3]

- *totipotent:* the fertilized egg, capable of independently giving rise to all embryonic and extraembryonic tissues;
- *pluripotent:* the inner cell mass of the blastocyst in the developing zygote and embryonic stem cells in culture, capable of giving rise to all embryonic cells and tissues;
- *multipotent fetal stem cells:* cells derived from the three embryonic germ layers (ectoderm, mesoderm and endoderm) that become more and more committed to generating particular cells as organs and tissues are formed.
- *multipotent adult stem cells:* thought to be tissue-specific and sometimes form only one type of cell (unipotent).

The notion that stem cells exist during embryonic development has long been accepted, but the thought that stem cells remain in various tissues after birth (adult stem cells) is relatively new. Based on observations that several tissues in the body (such as blood, skin and the gastrointestinal tract) undergo rapid renewal, scientists hypothesized that postnatal tissues must contain stem cells to initiate such replacement. The first definitive evidence came with the work of Till and McCulloch[5] on blood-forming (hematopoietic) stem cells in the 1960s. Many now believe that virtually every tissue in the body contains some type of stem cell, conjuring up thoughts of all types of strategies for tissue repair. Whether all of the candidates identified to date are true stem cells is debatable, on the basis of the strict definition given above. However, that is not to say that such cells are not of interest, in that even the more committed offspring of stem cells (progenitors) may have a role in cell-based tissue regeneration strategies. Furthermore, isolation and characterization of progenitor cells provide the opportunity to dissect more closely the mechanisms of tissue turnover and homeostasis that, when disrupted, lead to disease.

Plasticity: fact, fiction or fusion, and why do we need to know about it? Plasticity is the ability of a stem cell isolated from one tissue to "convert" to cells found in a different tissue, and sometimes even into cell types that originated from a completely different embryonic germ layer.[3] Recent reports of adult stem cell "plasticity" have generated a great deal of enthusiasm, as well as skepticism. However, if those reports are substantiated, adult stem cell plasticity would fulfill the tissue engineer's dream of isolating stem cells from easily accessible sources for regeneration of many different tissues. For example, researchers have reported that a single blood-forming stem cell, which originates from mesoderm, can give rise to nerve cells, which are derived from ectoderm, and to liver, lung and gastrointestinal epithelial cells, which originate from endoderm.[6] However, many reports have lacked rigorous proof of plasticity, and it is clear that in some cases, conversion of one cell type to another is due to fusion of a donor stem cell to a host cell. True plasticity can be demonstrated only by the ability of a single (clonogenic) cell to form cells of multiple different phenotypes and to be shown to function as those different cell types. The purist also would add that conversion must be more than an isolated phenomenon, but should occur at a high frequency and be persistent or stable.[3] Nonetheless, these intriguing studies warrant further consideration. Researchers have demonstrated that fusion of blood-forming stem cells with liver cells can have a biological benefit in the treatment of a liver disease,[7] perhaps owing to rejuvenation of the diseased liver cell nucleus by the stem cell cytoplasm. And even if true plastic conversion is a rare event, analyzing the way in which it occurs may provide clues as to how to better manipulate cell populations to increase its frequency.

SKELETAL (MESENCHYMAL) STEM CELLS

In the 1960s, Friedenstein and colleagues[8] and, later, Owen and Friedenstein[9] isolated and identified a population of cells from postnatal bone marrow that, when transplanted back into an animal, have the ability to form bone, cartilage, marrow fat cells and the stroma that supports blood formation (Figure 2). Their work, and that which came later (reviewed in Bianco and colleagues[10]), characterized these cells as components of the bone marrow stroma that were distinct from blood cells. On the basis of their in vivo source and their multipotent nature, Owen and Friedenstein[9] called these cells "bone marrow stromal stem cells" (BMSCs). More recently,

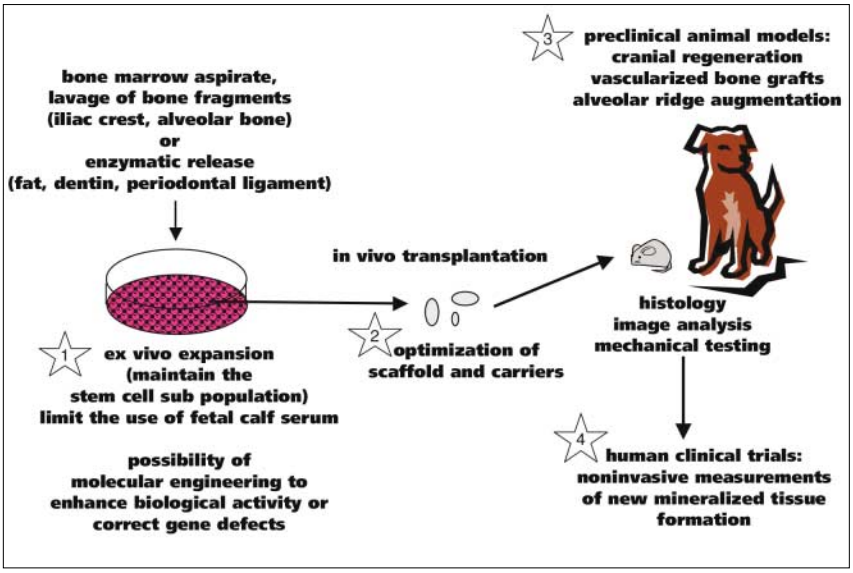

Figure 3. Steps in using postnatal stem cells in tissue engineering. Cells can be obtained from bone marrow aspirates or bone lavage of iliac crest or alveolar bone. Cells with the potential ability to form bone, cartilage, dentin and cementum can be isolated by enzymatic release of the cells from adipose tissue, dentin and periodontal ligament. **1.** Cells are expanded in culture to increase their numbers. During culture, they also may be genetically modified—for example, with lentiviral vectors. **2.** Once expanded, cells are transplanted in vivo in conjunction with appropriate scaffolds and carriers. In particular, hydroxyapatite/tricalcium ceramic particles are efficient in supporting the formation of a bone/marrow organ by bone marrow stromal cells (BMSCs). **3.** Subsequently, appropriate preclinical animal models are used to study efficacy by histology, imaging and mechanical testing. **4.** In human clinical trials, noninvasive assessments are preferable to biopsy.

others[11] have coined the term "mesenchymal stem cells." However, it must be noted that "mesenchyme" refers to an embryonic cell type that has the ability to give rise not only to connective tissue, but also to blood and blood vessels. To date, there is no evidence that any postnatal stem cell can give rise to both connective tissue and blood, with the possible exception of MAPCs (marrow-derived adult progenitor cells),[12] which are highly controversial and lack validation to date. Furthermore, numerous reports (reviewed in Barry and Murphy[13]) suggest that mesenchymal stem cells can be isolated from other connective tissues, but their similarity or dissimilarity to those isolated from bone marrow is not yet known. On the basis of these cells' tissue of origin and their ability to recreate all cell types associated with skeletal tissue, we have chosen to use the term "skeletal stem cells" (SSCs) for precision.[4,14]

Proof that an SSC does exist. In Friedenstein's[8,15,16] classic experiments, which have been repeated in numerous laboratories around the world, proof of the existence of an SSC came from experiments in which he plated single-cell suspensions of bone marrow at low density, such that a single bone marrow stromal cell—a cell that Friedenstein termed the "colony-forming unit–fibroblast" (CFU-F)—adhered and proliferated to form a colony (thus making the cell clonogenic) (Figure 2).[12] On in vivo transplantation with appropriate scaffolding, approximately one-fifth of the clones are able to form a complete bone/marrow organ.[15,17] When these clones are placed in a high-density culture (micromass or pellet) with appropriate growth factors, they are able to form cartilage[18] and therefore are multipotent (Figure 2). The remaining clones form only bone or fibrous tissue and are thought to represent more committed and differentiated cells. These experiments provide proof that the BMSC population contains a multipotent SSC.

What can SSCs really form? Investigators[16,17] have reported that SSCs (mesenchymal stem cells), like other adult stem cells, exhibit plasticity and are able to differentiate into cell types in addition to skeletal cells, such as muscle cells, nerve cells and cardiomyocytes (reviewed in Barry and Murphy[13] and Zipori[19]). For the most part, these studies examined the expression of several markers in vitro and after in vivo transplantation, but researchers have not yet conducted a rigorous assessment of functionality on a cellular level. Some studies (reviewed in Barry and Murphy[13] and Zipori[19]) have shown that the infusion of these cell populations either directly into an injured tissue or into the circulation can have a positive effect. This may not be due to a direct conversion of the cells to another cell type but, perhaps, to the fact that the cells secrete factors that encourage repair by local cells, as may be the case in the use of BMSCs to treat myocardial infarct.[3]

CURRENT APPROACHES TO TISSUE ENGINEERING

"Tissue engineering" is the general term for a number of ways by which tissue lost as a result of trauma and disease might be restored. Researchers can use cells alone (as in the case of bone marrow transplantation), but for dental and

craniofacial reconstruction, researchers more commonly use cells in combination with appropriate scaffolds and carriers that may or may not contain bioactive factors.[20,21] This approach should not be conceived of as being "one size fits all"; rather, it depends on the type of reconstruction that is desired, the state of the recipient tissue and the physiological status of the patient.

As in any rigorous biomedical study, tissue regeneration requires a systematic approach to identify tissues with the appropriate populations of stem cells and/or more committed progenitor cells, determine the best possible conditions for their ex vivo expansion, optimize the nature of the scaffolds and carriers, and develop appropriate preclinical animal models. Furthermore, outcomes and how they are measured must be defined strictly (Figure 3).

Cells: how do we handle them and where can we get them? To date, no markers exist that distinguish between SSCs and more committed BMSC types to purify SSCs directly from bone marrow.[14] But given the repeated demonstration of the high efficiency of populations of BMSCs (which contain SSCs) in regenerating a bone/marrow organ, as well as the need to generate large numbers of cells by ex vivo expansion for tissue regeneration, the inability to purify SSCs is not a critical limitation. The major concern during ex vivo expansion is the identification of culture conditions that maintain the important properties of SSC within the BMSC population. Normally, cells are grown in culture with nutrient medium that contains serum from fetal calves, which may represent a potential hazard in terms of transmitting viruses. Fortunately, human BMSCs can be grown in serum-free medium for up to four days before being harvested and are still quite viable and active in forming a bone/marrow organ.[22] This is important in view of the U.S. Food and Drug Administration recommendations/guidelines regarding the elimination of the use of fetal calf serum to the greatest extent possible when generating cells for human use.

Most preclinical studies have used cells from bone marrow aspirates of the iliac crest or washed out from large surgical specimens of bone.[23,24] Until recently, little attention has been paid to the specific type of bone used as a source of cells. However, the type of bone that is used for harvesting of marrow may be a significant issue. The axial/appendicular skeleton derives from the embryonic mesoderm, whereas bone of the craniofacial region develops from ectoderm. Little is known about how these different embryonic origins influence the activities of SSCs, but it has been noted that bone grafts from the ilium often are resorbed rapidly when placed in maxillary or mandibular bone.[25] A recent study suggests that there are differences between BMSCs from the iliac crest and BMSCs from the maxilla and mandible, based on the type of bone that they form on in vivo transplantation.[26] These results indicate that more study is needed to determine if BMSCs derived from axial/appendicular sources can, in fact, substitute for those of craniofacial origin.

Researchers also have reported that cells with SSC-like properties can be isolated from a variety of tissues other than bone marrow, including fat, peripheral blood and umbilical cord blood.[13] To date, the most promising of these extraskeletal sources appears to be fat.[27] Enzymatic treatment of fat tissue harvested by liposuction releases cells that are similar, but not identical in nature, to SSCs. Adipose-derived adult stem cells are able to differentiate into bone, cartilage and fat cell types in vitro. However, their properties have not been tested completely in vivo, and while they do appear to form bone, it is not known if they are capable of completely re-forming a bone/marrow organ. Likewise, researchers have identified cells in peripheral blood[28] and umbilical cord blood[29] that seem to exhibit similar properties. Circulating SSCs are exceedingly rare in humans, and their origin and function are unknown. Although somewhat more abundant, SSC-like cells from umbilical cord blood also are not isolated routinely. Periosteal tissue harvested from maxillary bone is a potentially attractive source for dental practice because of the ease of accessing and harvesting it. Preliminary data do, in fact, indicate that periosteal cells isolated from maxillary bone can be grown in culture easily and, when transplanted in vivo, form significant amounts of histology-proven bone (Riminucci and Bianco, unpublished data, January 2005).

BMSCs derived from healthy donors already are commercially available. It is thought that they do not express histocompatibility antigens and that they will not elicit an immune response if used in different people (allogenic)[13]; however, it is not clear that this is the case, especially once the cells become differentiated. It also is thought that BMSCs modulate the immune system,[13] and

although preclinical data are lacking, BMSCs are in trial for the treatment of graft-versus-host disease and Crohn's disease) for compassionate use in patients in whom all other treatments have failed. It is yet to be determined how efficacious allogenic cell preparations will be in any application.

Recently, using techniques that were developed for the characterization of BMSCs, a number of researchers[30-32] have reported the isolation of stem cells from the pulp of deciduous and permanent teeth and from the periodontal ligament (PDL). Dental pulp cells were shown to form dentin when transplanted in vivo, and cells derived from PDL formed cementum and a PDL-like structure.[26-28] Much is yet to be done with these heterogeneous cell populations to identify and characterize true stem cells and determine their relationship to SSCs. However, given the fact that deciduous teeth and third molars with attached PDL are available routinely, these tissues represent another source of autologous cells that would be of interest to the dental community for restorative procedures.

Scaffolds. The optimal properties of a scaffold or carrier for use in cell-based tissue engineering include the ability of the carrier to maintain the important characteristics of a stem cell and yet allow for the appropriate differentiation of its progeny, to provide adequate support for the developing tissue and then to be resorbed without the generation of toxic byproducts. Unfortunately, no scaffold currently available completely satisfies these criteria for regenerating bone or cartilage.

Investigators have tested a number of natural and synthetic polymers for bone regeneration, including collagen-based sponges, demineralized bone matrix, poly-L-lactic acid fibers and poly-L-glycolic acid fibers.[33] While these scaffolds have been found to be efficacious using nonhuman BMSCs, they do not well support bone formation by human BMSCs. To date, the most consistent bone formation by human BMSCs has been demonstrated with the use of synthetic hydroxyapatite/tricalcium phosphate (HA/TCP) ceramics.[34] However, these are not well-resorbed, but instead persist for long periods after transplantation. A number of HA/TCPs are available commercially that vary in both their HA/TCP composition and their manufacturing process. In general, the higher the proportion of HA, the more bone formation, whereas higher proportions of TCP lead to a more resorbable product but less bone formation. The optimal ratio has yet to be determined. Not all of the commercial products support the formation of a complete bone/marrow organ (M.H. Mankani and colleagues, unpublished data, March 2005). HA/TCP is osteoconductive; that is, it facilitates the unfolding of an inherent osteogenic character of cells without directing, per se, the biological performance of the cells. The lack of formation of a blood-supporting stroma with some HA/TCP preparations suggests that not all of them maintain the SSCs.

In addition to the composition of the HA/TCP, the size and shape of the scaffold also are important. In its first uses, porous HA/TCP was fashioned into blocks and BMSCs were introduced into the blocks by vacuum pressure. However, these types of transplants display bone formation only on the outermost surfaces and channels, owing to the inability of the cells to infiltrate the block completely. By using much smaller, roughly spherical particles of varying sizes with human BMSCs, scientists can achieve different patterns of bone formation with more or less marrow. Thus, the amount and organization of bone and the establishment of marrow can be controlled by the three-dimensional organization of the transplants, as dictated by the size of the particles and the spaces between them.[35]

The development of appropriate scaffolds for cartilage regeneration is far more problematic. Because cartilage does not contain blood vessels, the optimal scaffold would need to allow nutrient exchange but prevent the ingrowth of blood vessels. For that purpose, various types of hydrogels are under development. Furthermore, the scaffold likely would need to include factors that prevent the cartilage from undergoing hypertrophy.[36] Hypertrophy is the process by which most of the cartilage formed during fetal life becomes calcified and then is replaced by bone, and the factors that prevent hypertrophy in cartilages that persist are not well-known. In the craniofacial region, in addition to the hyaline cartilages of the nasal septum and the ear, the articular surface of the mandibular condyle represents yet another challenge. This fibrocartilage is distinctly different from other cartilages, and the factors that regulate its formation and maintenance are not well-understood. Determining how to manipulate human BMSCs to reconstruct this type of tissue will require extensive investigation.[37]

Growth factors. Researchers have invested a great deal of effort in identifying growth factors

that control the development of bone and its marrow and in using them to enhance bone regeneration. It has long been known that bone matrix contains an active ingredient, bone morphogenetic protein (BMP), that can induce bone formation outside of the skeleton when introduced in combination with an appropriate scaffold. The induced bone is formed via a cartilage anlage that is remodeled into bone, but induced bone ultimately can disappear owing to the eventual degradation of the BMP. In skeletal defects, BMP administration does appear to hasten the repair process. For this reason, efforts are under way to use molecular techniques to enhance BMP production by cells before their use in tissue engineering.[38] However, it is not clear that this is necessary or desirable for human BMSCs, which make a number of BMPs on their own, or whether additional BMP production would lead to unwanted bone formation outside of the transplant.

In addition to BMPs, other factors—such as transforming growth factor–beta, fibroblast growth factor, insulinlike growth factor–1, platelet-derived growth factor, vascular endothelial growth factor and prostaglandin E2—all have been demonstrated to influence bone formation and vascular ingrowth into various scaffoldings.[38] These factors do influence the activity of local stem cell populations, but in many clinically relevant cases, the local population has been obliterated owing to trauma or disease, and growth factors, even when used with a scaffold, most likely will be unable to regenerate vast areas of bone. Thus, growth factors likely will be most useful in combination with cell-based therapies.

CELL-BASED APPLICATIONS TO OROFACIAL RECONSTRUCTION

An estimated 1,600,000 bone grafts are performed every year to regenerate bone lost to trauma and disease, of which 6 percent (96,000) are craniomaxillofacial in nature.[39] These procedures rely on autologous bone grafting, devitalized allogenic grafting (using bone from a bone bank) and the use of natural and synthetic osteoconductive biomaterials. Autologous bone grafting is limited by the amount of bone that can be harvested and by donor site morbidity, and allogenic bone often is destroyed rapidly. Biomaterials can be useful in many cases, but the long-term outcome relies heavily on their ability to encourage local cells to completely regenerate a defect, and their effects often are not enduring. It is clear that new techniques are needed to more predictably restore function and form, especially in the craniofacial region.

Given the tools that are immediately in hand (ex vivo expanded human BMSCs and HA/TCP ceramic particles), researchers have developed a number of applications in preclinical animal models for the repair of, as examples, segmental defects that would never heal on their own ("critical size" defects) and the development of bone rudiments with intact blood vessels. These procedures have direct application for restoration of bone defects in the craniofacial region. Researchers have developed other applications for restoration of alveolar ridge height, but primarily with the use of scaffolds alone or in conjunction with BMPs. Long-bone defects have been treated in a number of patients with BMSCs and HA/TCP,[40] and a small number of clinical trials for both long-bone defects and for alveolar ridge augmentation are under way.

Direct orthotopic transplantation into segmental defects. Investigators have developed a number of animal models of segmental defects in mice,[41] dogs[24] and sheep.[23] In sheep, ceramic blocks loaded with BMSCs were found to completely heal long-bone defects. In mice and dogs, investigators created critical-size defects in the cranium and filled them with ex vivo expanded BMSCs either in collagen sponges or in association with HA/TCP (Figure 4A). In both cases, the defects healed completely, and the newly formed bone integrated into the margin (Figure 4B). Researchers have succeeded in using porous silk fibroin scaffolds as a support for growth and differentiation of BMSCs in a bioreactor before transplantation in a cranial critical-size defect in mice.[42]

Alveolar ridge augmentation. Restoration of alveolar ridge height is of utmost concern to practicing dentists in trying to prevent the loss of a tooth due to bone destruction induced by periodontal disease, and in maintaining the ability of edentulous patients to wear dentures. Appropriate ridge height also is essential for the placement and long-term retention of dental implants. Standard practice involves the use of autologous or allogeneic bone grafts, or ceramics, both with and without growth factors, but the outcomes are variable. In animal models, BMSCs used in conjunction with HA/TCP have been successful in building alveolar bone,[43] and a number of small

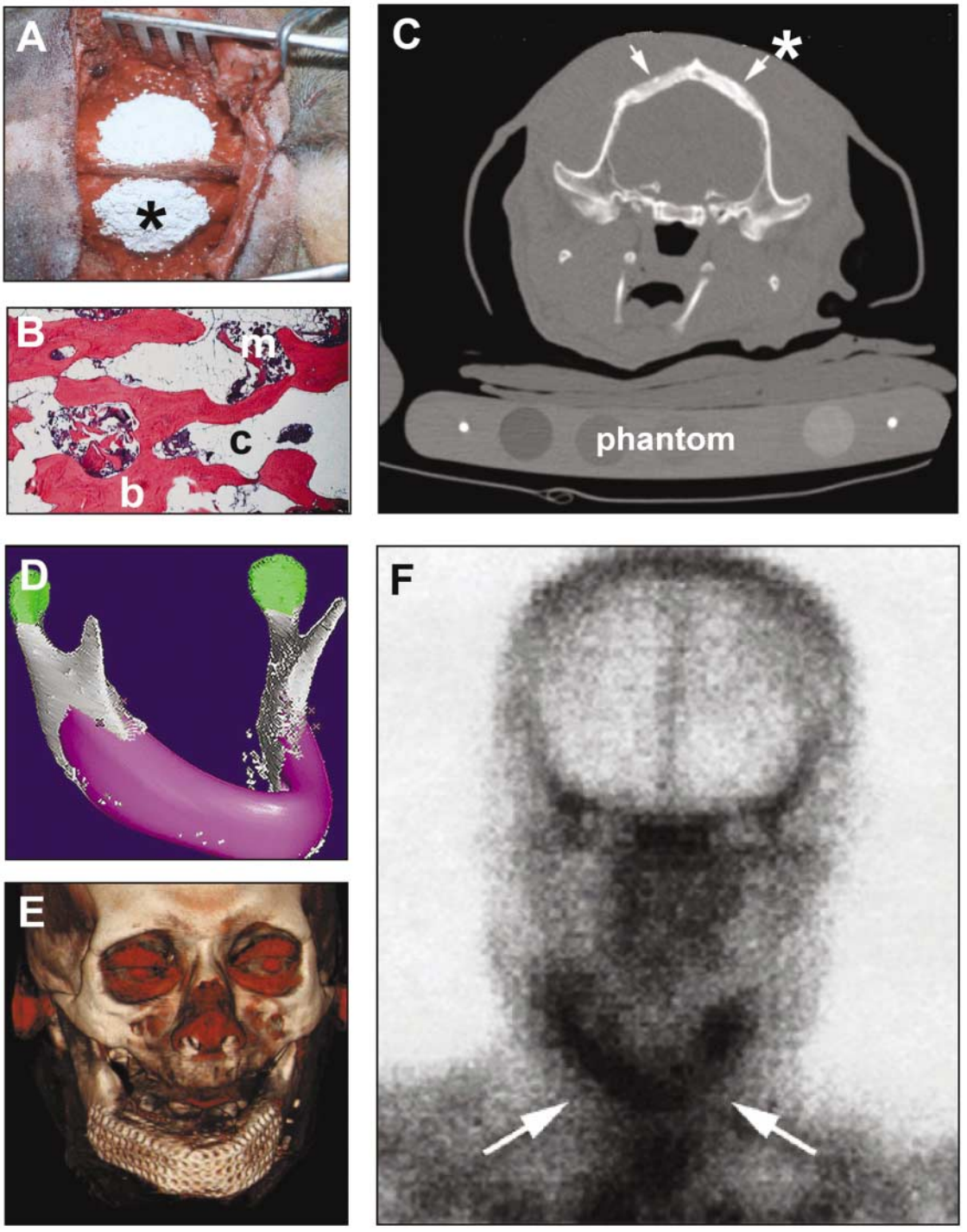

Figure 4. The use of bone marrow and its stromal cells (BMSCs) for craniofacial regeneration. Recent studies have shown that BMSCs are capable of fully regenerating large calvarial defects in dogs that never would heal on their own **(A-C)**. Defects were made in the calvaria **(A)**, with one side filled with hydroxyapatite/tricalcium phosphate (HA/TCP) alone and one side with HA/TCP including BMSCs (marked with an asterisk). After six months **(B)**, exuberant bone formation was noted (b) on the surfaces of the carrier particles (c), along with formation of foci of marrow (m). Quantitative computerized tomography (qCT) **(C)** could be used to examine the defects (arrows), and to distinguish new bone formation in the defect with BMSCs (*) in spite of the presence of HA/TCP. (Figures 4A and 4C modified from Mankani and colleagues[24] with permission of the American Journal of Pathology.) Using bone marrow, a vascularized bone graft also has been used to reconstruct a large mandibular defect in a patient **(D-F)**. First, CT was used to generate a model of the defect **(D)** and used as a template for the construction of a titanium cage, which was filled with bone marrow, devitalized bone chips and bone morphogenetic protein (BMP). The cage was then placed into the latissimus dorsi muscle, and after seven weeks, was removed, along with muscle and blood vessels and moved into the mandible, where the blood vessels were attached to the external carotid artery and the cephalic vein **(E)**. The viability of the vascularized bone graft was confirmed by bone scan **(F)**. (Figures 4D-F reprinted from Warnke and colleagues[48] with permission of Elsevier.)

studies in human patients have used BMSCs along with allogenic bone fragments[44] or with platelet-rich plasma,[45] as well as with another ceramic scaffold, beta-calcium phosphate.[46] With further refinement, these types of procedures would mark a major advancement in dental reconstruction.

Vascularized bone grafts. It has long been a practice of plastic and orthopedic surgeons to isolate flaps of bone with intact blood vessels (usually from the fibula, a non–weight-bearing bone) to reconstruct bone in a recipient site that is morbid because of infection, radiation injury or extensive damage to local blood vessels. However, this practice results in a substantial increase in risk and discomfort to the patient, as well as donor-site morbidity. To circumvent this, researchers have developed methods to generate vascularized bone grafts by placing BMSCs in collagen sponges, which then are wrapped around an artery and vein. The collagen sponge containing cells subsequently is wrapped with Teflon (Dupont Teflon, Wilmington, Del.) to prevent blood vessel ingrowth from the side (collateral ingrowth). After several weeks, these bone rudiments are found to be perfused entirely by the artery and vein that they surround, and they then can be moved to another site where the blood vessels can be reattached to existing blood vessels in the margins of the recipient site.[47]

It is exactly this type of procedure that clinical researchers used recently to reconstruct part of a mandible in a patient who had undergone extensive tumor resection.[48] In this case, researchers used computerized tomographic (CT) scanning to model the jaw (Figure 4D) and fabricated a custom-made titanium mesh cage to match the dimensions of the defect, filled it with bovine bone powder, and infiltrated it with the patient's own bone marrow (rather than ex vivo expanded BMSCs) and bone morphogenetic protein (BMP). They then placed the cage in the highly vascularized latissimus dorsi muscle. Seven weeks later, they removed the cage, along with part of the muscle containing the thoracodorsal artery and vein, and then moved it into the mandible (Figure 4E). Here, they attached the blood vessels to the external carotid artery and cephalic vein, which they relocated from the patient's upper arm into the neck. The researchers,[45] using bone scintigraphy, found the graft to be biologically active (Figure 4F). Long-term results for this patient have yet to be reported. In this case, the researchers used bone marrow, and one can envision that the use of ex vivo expanded BMSCs could hasten the development of bone, thereby shortening the period in which the transplant needs to be grown in the donor site.

OUTCOMES: ARE WE REALLY DOING ANYTHING GOOD?

One of the major aspects in cell-based tissue engineering is development of appropriate outcome measures by which to determine if, in fact, new bone is generated and, ultimately, if the biomechanical properties of the newly formed bone are sufficient to restore form and function. The most direct measure by which to assess new bone formation is histological analysis. However, biopsy of transplants in patients is less than desirable, and noninvasive methods are needed to evaluate the outcomes of BMSC transplantation into human patients.

Histology. In any preclinical model, histological examination of newly formed tissues is essential. Bone is highly identifiable through standard hematoxylin and eosin (H&E) staining of decalcified paraffin sections. In addition, bone is fluorescent when H&E-stained sections are illuminated with ultraviolet light, whereas most other connective tissues are not.[49] In nondecalcified, plastic-embedded sections, von Kossa's staining demonstrates mineralized tissue (black), and Goldner's trichrome distinguishes between mineralized tissue (green) and unmineralized osteoid (red). Researchers can distinguish woven bone from lamellar bone in paraffin or plastic sections with polarized light microscopy.

Radiography. In transplants that are generated by BMSCs in collagen sponges and other nonmineralized scaffolds or carriers, standard radiography can be quite useful in assessing new bone formation. Unfortunately, to date, these types of scaffolds do not support good bone formation by human BMSCs. Assessment of bone formation by radiography, in current transplants used for human BMSCs is not feasible owing to the radiopacity of the HA/TCP particles used as scaffolds. However, this form of measurement may become possible in the future if researchers develop nonmineral-containing scaffolds that support bone formation by human BMSCs.

Bone scan. Bone scintigraphy relies on the uptake of 99mTc-labeled methylene diphosphonate by metabolically active bone, and it is used to detect disease activity in a variety of condi-

tions.[50] As mentioned above, investigators have used this technique to determine the viability of a mandibular transplant, and it may be useful in following the course of bone formation at different times after transplantation of human BMSCs with HA/TCP particles—in large defects, at least.

Quantitative CT (qCT). In standard CT images, it is not possible to distinguish HA/TCP from newly formed bone. However, it is possible with specialized software (Mindways Software, Austin, Texas) and a standard clinical CT scanner to perform qCT to measure the bone mineral density of a volume of tissue in comparison with phantoms of known density. qCT also provides the opportunity for three-dimensional reconstruction of complex regions of interest, which is not possible with plain radiographs or bone scans. Recently, investigators demonstrated that qCT can distinguish between constructs in which good bone formation can be observed histologically as compared with those in which little or no bone formation can be observed (Figure 4B).[51] As new software and scanners become increasingly refined, this technique should be valuable as a noninvasive evaluation tool.

Mechanical testing. There are no direct noninvasive measures of the mechanical properties of newly formed bone. Determination of mechanical strength relies on measurements of bone architecture (cortical thickness, cortical diameter, periosteal surface, endosteal surface, trabecular thickness, trabecular spacing) as can be made by noninvasive imaging techniques, which by current instruments are at low resolution only. Clinicians are unable to directly measure parameters of strength in newly formed bone in a patient, other than by watching to see if normal function is restored. Therefore, any preclinical study must incorporate in its design a comparison of these imaging parameters with mechanical and material properties that typically are performed ex vivo.[52]

FUTURE CHALLENGES

Molecular engineering. In the case of genetic diseases, when a protein is missing or defective and causes craniofacial abnormalities, it would be beneficial to engineer a patient's own SSCs to replace the defective gene and restore normal cell function (a process known as "gene therapy"). The two basic methods for molecular engineering are based on nonviral and viral techniques. Owing to the fact that nonviral methods are fairly inefficient, the use of viral vectors has been the predominant approach in this area. Viral vectors are viruses engineered to incorporate a given DNA sequence to elicit or control the activity of a particular gene or regulatory pathway. On the basis of the biology of viral vectors, two broad categories are defined:

- those that do not incorporate into the host cell genome and therefore can function only over the short term, such as adenoviruses;
- those that do incorporate into the cell's genome (such as oncoretroviral vectors and lentiviral vectors) and thus can, in principle, direct gene expression for the life span of the cell and its progeny.

Researchers have envisioned molecular engineering mostly for the delivery of osteoinductive factors (BMPs).[53] In many of these approaches, investigators have used adenoviral vectors, which do not incorporate into the cell's genome and do not infect target cells with high efficiency. The ultimate utility of this approach is less than clear, because bone formation by SSCs does not require additional BMPs and because, vice versa, any number of cells other than SSCs (any fibroblast, in fact) could be used for genetic engineering and local BMP delivery. More interesting seems the prospect of permanently modifying SSCs with retroviral vectors. Lentiviral vectors, in particular, have been proven to be efficient and neutral with respect to cell function and represent a viable option.[54] In this respect, these tools will provide not only long-term production of a desired protein, but also the opportunity to design constructs able to modulate, or silence, the expression of a given gene. This will find a potential application in many genetic diseases of the skeleton affecting the craniofacial bones.

Percutaneous delivery. In many situations in the craniofacial region, introduction of cells and scaffolds requires open surgery. However, in some situations in which defects are highly localized, such as in isolated cysts or cleft defects in the jawbones, patients could benefit from the delivery of BMSCs in an appropriate semisolid carrier via injection directly into the defect through the epithelial surface. Such a carrier would have all of the criteria for scaffolds outlined earlier and would hold the cells within the defect. A number of injectable carriers are available (calcium phosphate and calcium sulfate cements, polylactide, polyglycolide, and mixtures of polylactide and polyglycolide, which can be used to deliver osteogenic factors.[55] But to date, none has

been shown to both maintain viability and support formation of bone by human BMSCs. In other studies, investigators used BMSCs along with platelet-rich plasma with promising results (reviewed in Yamada and colleagues[56]); however, this type of procedure provides little mechanical support. With further development, being able to directly deliver cells without open surgery would be of major advantage.

Other types of craniofacial reconstruction. Restoring bone is essential for promoting functionality of dental implants, and the future promises even more exciting possibilities for dental practice. Potential stem cells and more committed progenitors have been identified in unerupted tooth buds, or the dental pulp of deciduous and permanent teeth, and in periodontal ligament (PDL).[30-32] These cells provide the prospect of further restoring dentally relevant tissues such as dentin, cementum and PDL. Today's dental implants rely on the ability of bone to interface with metal (usually titanium). This interface may be improved by the development of cementum on the implant surface, along with re-establishment of a PDL between the newly formed cementum and the alveolar bone.

One also can imagine that if an enamellike biomaterial can be fabricated in the shape of a tooth to be replaced, dentin can be re-formed within that "cap" and placed in a vascular bed, much in the same way as described above for a vascularized bone graft. Once consolidated, this "viable" tooth then could be moved into the jaw with vasculature intact for further regeneration of cementum and PDL.[57] But what has really captured the imagination of dental scientists is the possibility to recreate a tooth bud that then can be placed in the jaw to develop, grow and erupt on its own. A recent study showed that cells isolated from unerupted tooth buds have the ability to reorganize into "mini-teeth" when transplanted within a carrier in vivo.[58] In addition, it also can be envisioned that tooth buds could be formed by introducing mesenchyme from nondental sources, or by using different types of stem cells (such as bone marrow–derived or neural stem cells) in conjunction with oral epithelium. One study using a similar approach generated tooth bud–like structures in rodents.[59]

CONCLUSIONS

At this time, science clearly indicates that the use of stem cells for regeneration, reconstruction or repair of bone is feasible in principle. Substantial advances have been made in our ability to handle skeletal stem cells in the laboratory, and to exploit their inherent potential for building bone. Translation of these advances into clinical practice will occur. How rapidly this will happen, however, depends on solving technical problems that still are significant. The kind of scaffold, the source of cells, the type of in vitro culturing, the surgical procedure to be used—all require careful consideration. The endeavor is clearly multidisciplinary in nature, and the practicing dental surgeon has a critical role in it. Playing this role in the most effective way requires awareness of the huge potential associated with the use of stem cells in a clinical setting, as well as proper understanding of the related problems. ■

The authors acknowledge the support of the Division of Intramural Research, National Institute of Dental and Craniofacial Research, Intramural Research Program, National Institutes of Health (1 Z01 DE000380 and 1 Z01 DE000649) (to Dr. Robey), and Telethon Fondazione ONLUS (grant GGP04263), the Ministry of Education, University and Research (MIUR grants RBAU0155SP, RBIN04CLZW_00) and the European Commission FP6 Research Funding Programme (Genostem, LSHB-CT-2003-503161) (to Dr. Bianco).

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