

Bone Marrow Stromal Stem Cells: Nature, Biology, and Potential Applications

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ABSTRACT

Bone marrow stromal cells are progenitors of skeletal tissue components such as bone, cartilage, the hematopoiesis-supporting stroma, and adipocytes. In addition, they may be experimentally induced to undergo unorthodox differentiation, possibly forming neural and myogenic cells. As such, they represent an important paradigm of

post-natal nonhematopoietic stem cells, and an easy source for potential therapeutic use. Along with an overview of the basics of their biology, we discuss here their potential nature as components of the vascular wall, and the prospects for their use in local and systemic transplantation and gene therapy. *Stem Cells* 2001;19:180-192

INTRODUCTION

The post-natal bone marrow has traditionally been seen as an organ composed of two main systems rooted in distinct lineages—the hematopoietic tissue proper and the associated supporting stroma. The evidence pointing to a putative stem cell upstream of the diverse lineages and cell phenotypes comprising the bone marrow stromal system has made marrow the only known organ in which two separate and distinct stem cells and dependent tissue systems not only coexist, but functionally cooperate. Originally examined because of their critical role in the formation of the hematopoietic microenvironment (HME), marrow stromal cells later came to center stage with the recognition that they are the stem/progenitor cells of skeletal tissues. More recent data pointing to the unexpected differentiation

potential of marrow stromal cells into neural tissue or muscle grant them membership in the diverse family of putative somatic stem cells. These cells exist in a number of post-natal tissues that display transgerminal plasticity; that is, the ability to differentiate into cell types phenotypically unrelated to the cells in their tissue of origin.

The increasing recognition of the properties of marrow stromal cells has spawned a major switch in our perception of their nature, and ramifications of their potential therapeutic application have been envisioned and implemented. Yet, several aspects of marrow stromal cell biology remain in question and unsettled throughout this evolution both in general perspective and in detail, and have gained further appeal and interest along the way. These include the identity, nature, developmental origin and in vivo function of marrow stromal

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cells, and their amenability to *ex vivo* manipulation and *in vivo* use for therapy. Just as with other current members of the growing list of somatic stem cells, imagination is required to put a finger on the seemingly unlikely properties of marrow stromal cells, many of which directly confront established dogmas or premature inferences made from other more extensively studied stem cell systems.

COLONY FORMING UNIT-FIBROBLAST (CFU-F) AND THEIR PROGENY

Alexander Friedenstein, Maureen Owen, and their coworkers were the first to utilize *in vitro* culture and transplantation in laboratory animals, either in closed systems (diffusion chambers) or open systems (under the renal capsule, or subcutaneously) to characterize cells that compose the physical stroma of bone marrow [1-3]. Because there is very little extracellular matrix present in marrow, gentle mechanical disruption (usually by pipetting and passage through syringe needles of decreasing sizes) can readily dissociate stroma and hematopoietic cells into a single-cell suspension. When these cells are plated at low density, bone marrow stromal cells (BMSCs) rapidly adhere and can be easily separated from the nonadherent hematopoietic cells by repeated washing. With appropriate culture conditions, distinct colonies are formed, each of which is derived from a single precursor cell, the CFU-F.

The ratio of CFU-F in nucleated marrow cells, as determined by the colony-forming efficiency (CFE) assay [4], is highly dependent on the culture conditions, and there is a great deal of variability in the requirements from one animal species to another. In rodents, irradiated marrow feeder cells are absolutely required in addition to selected lots of serum in order to obtain the maximum number of assayable CFU-F (100% CFE), whereas CFE is feeder cell-independent in humans [5]. The mitogenic factors that are required to stimulate the proliferation of CFU-F are not completely known at this time, but do at least include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor, transforming growth factor- β , and insulin-like growth factor-1 [6, 7]. Under optimal conditions, multi-colony-derived strains (where all colonies are combined by trypsinization) can undergo over 25 passages *in vitro* (more than 50 cell doublings), demonstrating a high capacity for self-replication. Therefore, billions of BMSCs can be generated from a limited amount of starting material, such as 1 ml of a bone marrow aspirate. Thus, the *in vitro* definition of BMSCs is that they are rapidly adherent and clonogenic, and capable of extended proliferation.

HETEROGENEITY OF THE BMSC POPULATION

The heterogeneous nature of the BMSC population is immediately apparent upon examination of individual

colonies. Typically this is exemplified by a broad range of colony sizes, representing varying growth rates, and different cell morphologies, ranging from fibroblast-like spindle-shaped cells to large flat cells. Furthermore, if such cultures are allowed to develop for up to 20 days, phenotypic heterogeneity is also noted. Some colonies are highly positive for alkaline phosphatase (ALP), while others are negative, and a third type is positive in the central region, and negative in the periphery [8]. Some colonies form nodules (the initiation of matrix mineralization) which can be identified by alizarin red or von Kossa staining for calcium. Yet others accumulate fat, identified by oil red O staining [9], and occasionally, some colonies form cartilage as identified by alcian blue staining [10].

Upon transplantation into a host animal, multi-colony-derived strains form an ectopic ossicle, complete with a reticular stroma supportive of myelopoiesis and adipocytes, and occasionally, cartilage [8, 11]. When single colony-derived BMSC strains (isolated using cloning cylinders) are transplanted, a proportion of them have the ability to completely regenerate a bone/marrow organ in which bone cells, myelopoietic stroma, and adipocytes are clonal and of donor origin, whereas hematopoiesis and the vasculature are of recipient origin [7] (Fig. 1). These results define the "stem" cell nature of the original CFU-F from which the clonal strain was derived. However, they also confirm that not all of the clonogenic cells (those cells able to proliferate to form a colony) are in fact multipotent stem cells. It must also be noted that it is the behavior of clonal strains upon transplantation, and not their *in vitro* phenotype, that provides the most reliable information on the actual differentiation potential of individual clones. Expression of osteogenic, chondrogenic, or adipogenic phenotypic markers in culture (detected either by mRNA expression or histochemical techniques), and even the production of mineralized matrix, does not reflect the degree of pluripotency of a selected clone *in vivo* [12]. Therefore, the identification of "stem" cells among stromal cells is only done *a posteriori* and only by using the appropriate assay. In this respect, chondrogenesis requires an additional comment. It is seldom observed in open transplantation assays, whereas it is commonly seen in closed systems such as diffusion chambers [11], or in micromass cultures of stromal cells *in vitro* [13], where locally low oxygen tensions, *per se*, permissive for chondrogenesis, are attained [14]. Thus, the conditions for transplantation or even *in vitro* assays are critical determinants of the range of differentiation characteristics that can be assessed.

The ability to isolate the subset of marrow stromal cells with the most extensive replication and differentiation potential would naturally be of utmost importance for both theoretical and applicative reasons. This requires definitive linkage

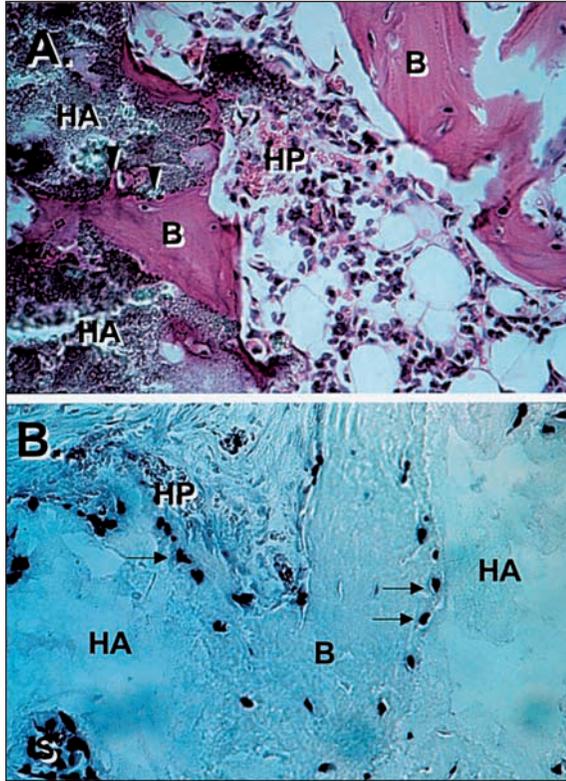


Figure 1. Transplantation of ex vivo-expanded human BMSC into the subcutis of immunocompromised mice. A) Multi-colony and some single colony-derived strains attached to particles of hydroxyapatite/tricalcium phosphate ceramic (HA) form a complete bone/marrow organ composed of bone (B) encasing hematopoietic marrow (HP). B) The bone (B) and the stroma (S) are of human origin as determined by in situ hybridization using a human specific alu sequence as probe, while the hematopoietic cells are of recipient origin.

of the multipotency displayed in transplantation assays with a phenotypic trait that could be assessed prior to, and independently of, any subsequent assays. Several laboratories have developed monoclonal antibodies using BMSCs as immunogen in order to identify one or more markers suitable for identification and sorting of stromal cell preparations [15-18]. To date, however, the isolation of a “pure” population of multipotent marrow stromal stem cells remains elusive. The nearest approximation has been the production of a monoclonal antibody, Stro-1, which is highly expressed by stromal cells that are clonogenic (Stro-1^{bright}), although a certain percentage of hematopoietic cells express low levels of the antigen (Stro-1^{dull}) [19]. In principle, the use of the same reagent in tissue sections would be valuable in establishing in vivo-in vitro correlation, and in pursuing the potential microanatomical niches, if not anatomical identity, of the cells that are clonogenic. The Stro-1 reagent has limited application in fixed and paraffin-embedded tissue. However, preliminary

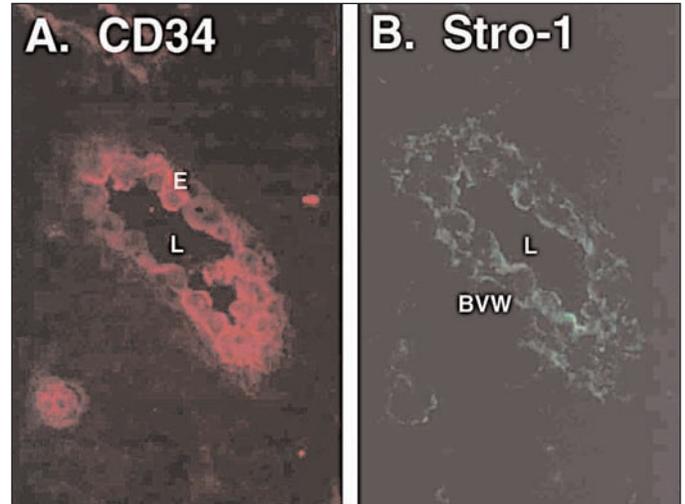


Figure 2. Immunolocalization of the Stro-1 epitope in the microvasculature of human thymus. A) CD34 localizes to endothelial cells (E) forming the lumen (L) of the blood vessel. B) Stro-1 localizes not only to endothelial cells, but also the perivascular cells of the blood vessel wall (BVW).

data using frozen sections suggest that the walls of the microvasculature in a variety of tissues are the main site of immunoreactivity (Fig. 2), a finding of potentially high significance (see below).

Freshly isolated Stro-1^{bright} cells and multi-colony-derived BMSC strains, both of which contain but are not limited to multipotent stromal stem cells, have been extensively characterized for a long list of markers expressed by fibroblasts, myofibroblasts, endothelial cells, and hematopoietic cells in several different laboratories [20-24]. From these studies, it is apparent that the BMSC population at large shares many, but not all, properties of fibroblastic cells such as expression of matrix proteins, and interestingly, some markers of myofibroblastic cells, notably, the expression of α -smooth muscle actin (α -SMA) and some characteristics of endothelial cells such as endoglin and MUC-18. It has been claimed that the true “mesenchymal stem cell” can be isolated using rather standard procedures, and characterized using a long list of indeterminate markers [23]. However, in spite of this putative “purification” and extensive characterization, the resulting population was no more “pure” than multi-colony-derived strains isolated by simple, short-term adherence to plastic; the resulting clones displayed varying degrees of multipotentiality. Furthermore, the pattern of expressed markers in even clonal strains that are able to completely regenerate a bone/marrow organ in vivo is not identical, and changes as a function of time in culture. These results indicate that identifying the “phenotypic fingerprint” of a stromal stem cell may well be like shooting at a moving target, in that they seem to be

constantly changing in response to their microenvironment, both in vitro and in vivo.

POST-NATAL MARROW STROMAL CELLS AS CELLS OF THE VASCULAR WALL

The primitive marrow stroma is established in development through a complex series of events that takes place following the differentiation of primitive osteogenic cells, the formation of the first bone, and the vascular invasion of bone rudiments [25]. This intimate relationship of the stromal cells with the marrow vascularity is also found in the adult marrow. In the post-natal skeleton, bone and bone marrow share a significant proportion of their respective vascular bed [26]. The medullary vascular network, much like the circulatory system of other organs, is lined by a continuous layer of endothelial cells and subendothelial pericytes [27]. In the arterial and capillary sections of this network, pericytes express both ALP (Fig. 3B, C, D, F, G) and α -SMA (Fig. 3E), both of which are useful markers for their visualization in tissue sections. In the venous portion, cells residing on the abluminal side of the endothelium display a "reticular" morphology, with long processes emanating from the sinus wall into the adjacent hematopoietic cords where they establish close cell-cell contacts, that convey microenvironmental cues to maturing blood cells. These particular adventitial reticular cells express ALP (Fig. 3G) but not α -SMA under normal steady-state conditions (Fig. 3H). In spite of this, but in view of their specific position along with the known diversity of pericytes in different sites, organs and tissues [28], reticular cells can be seen as bona fide specialized pericytes of venous sinusoids in the marrow. Hence, phenotypic properties of marrow pericytes vary along the different sections of the marrow microvascular network (arterial/capillary versus post-capillary venous sinusoids). In addition, adventitial reticular cells of venous sinusoids can accumulate lipid and convert to

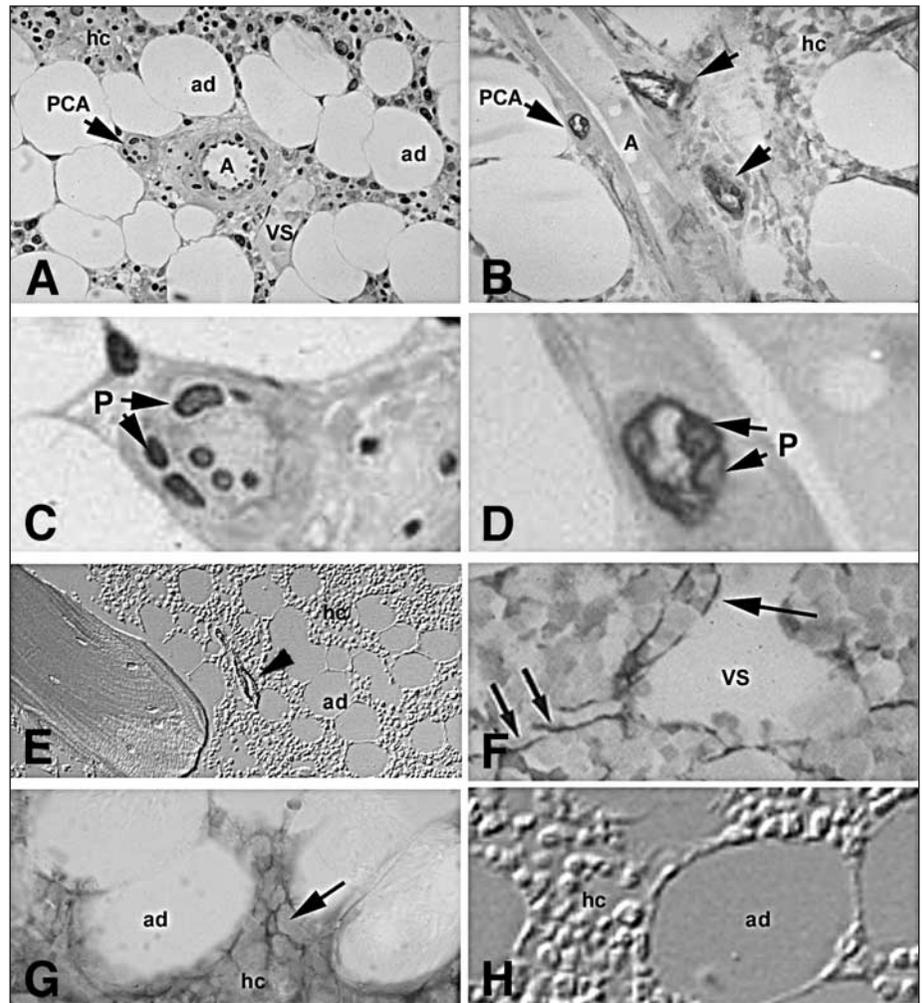


Figure 3. Anatomical and immunohistological relationship of marrow stromal cells to marrow pericytes. A) Marrow vascular structures as seen in a histological section of human adult bone marrow. hc = hematopoietic cells; ad = adipocytes; a = artery; VS = venous sinusoid; PCA = pre-capillary arteriole. Note the thin wall of the venous sinusoid. B) Semi-thin section from low-temperature processed glycol-methacrylate embedded human adult bone marrow reacted for ALP. Arrows point to three arterioles emerging from a parent artery (A). Note that while there is no ALP activity in the wall of the large size parent artery, a strong reaction is noted in the arteriolar walls. C, D) Details of the arterioles shown in A and B. Note that ALP activity is associated with pericytes (P). E) Section of human adult bone marrow immunolabeled for α -SMA. Note the reactivity of an arteriolar wall, and the complete absence of reactivity in the hematopoietic cords (hc) interspersed between adipocytes (ad). F) Detail of the wall of a marrow venous sinusoid lined by thin processes of adventitial reticular cells (venous pericytes). Note the extension of cell processes apparently away from the wall of the venous sinusoid (vs) and into the adjacent hematopoietic cord ALP reaction. G, H) High power views of hematopoietic cords in sections reacted for ALP (G) and α -SMA (H). Note the presence of ALP activity identifying "reticular" cells, and the absence of labeling for α -SMA.

adipocytes, and they do so mainly under two circumstances: A) during growth of an individual skeletal segment when the expansion of the total marrow cavity makes available space in excess of what is required by hematopoietic cells, or B) independent of growth, when there is an abnormal or age-related numerical reduction of hematopoietic cells thereby making space redundant [29-31].

The ability of reticular cells to convert to adipocytes makes them a unique and specialized pericyte. Production of

a basement membrane by adipocytes endows the sinus with a more substantial basement membrane, likely reducing the overall permeability of the vessel. Furthermore, the dramatic increase in cell volume through the accumulation of lipid during adipose conversion collapses the lumen of the sinus. This may exclude an individual sinus from the circulation without causing its irreversible loss. In general, the loss of pericyte coating on a microvessel is associated with vessel regression by apoptosis, while a normal pericyte coating is thought to stabilize them and prevent vessel pruning [32]. Adipose conversion is thus a mechanism whereby the size and permeability of the overall sinusoidal system is reversibly regulated in the bone marrow. Not surprisingly, regions of bone marrow that are hematopoietically inactive are filled with fat.

Given the similar location of pericytes and stromal cells, the significance of α -SMA expression, a marker of smooth muscle cells, in marrow stromal cells takes on new meaning, although its expression is variable, both in vitro and in vivo. α -SMA expression is commonly observed in nonclonal, and some clonal cultures of marrow stromal cells [33], where it appears to be related to phases of active cell growth [34], and may reflect a myoid differentiation event, at least in vitro [35]. However, the phenotype of α -SMA-expressing stromal cells in culture resembles that of pericytes and subintimal myoid cells rather than that of true smooth muscle cells [35]. In the steady-state normal bone marrow, α -SMA expressing stromal cells other than those forming the pericyte/smooth muscle coats of arteries and capillaries are not seen. In contrast, α -SMA⁺ stromal cells not associated with the vasculature are commonly observed in the fetal bone marrow [36, 37], that physically grows together with the bone encasing it. α -SMA⁺ marrow stromal cells are likewise seen in conjunction with a host of hematological diseases [37], and in some bone diseases, such as hyperparathyroidism [37] and fibrous dysplasia (FD) of bone (*Riminucci and Bianco*, unpublished results). In some of these conditions, these cells have been interpreted as myofibroblasts [34, 37]. More interestingly, at least some of these conditions also feature an increased vascularity, possibly related to angiogenesis [38], and an increased number of CFU-F, quantitated as discussed above (*Bianco, Kuznetsov, Robey*, unpublished results). Taken together, these observations seem to indicate that α -SMA expression in extravascular marrow stromal cells (other than arterial/capillary pericytes) is related to growth or regeneration events in the marrow environment, which is in turn associated with angiogenesis.

Angiogenesis in all tissues involves the coordinated growth of endothelial cells and pericytes. Nascent endothelial tubes produce EGF and PDGF-B, which stimulate the growth and migration of pericytes away from the subintimal myoid cell layer of the vascular section. A precise ligand-receptor

expression loop of PDGF-B produced by endothelial cells and expression of the cognate receptor on pericytes regulates the formation of a pericyte coating and its occurrence in physical continuity with the nascent vascular network [39]. Interestingly, PDGF-receptor beta and EGF receptor are two of the most abundant tyrosine kinase growth factor receptors in BMSCs, and PDGF-B and EGF have been found to stimulate proliferation of BMSCs [6, 40], indicating a physiological similarity between pericytes and BMSCs.

In bone, as in any other organ, angiogenesis is normally restricted to phases of developmentally programmed tissue growth, but may reappear in tissue repair and regeneration or proliferative/neoplastic diseases. During normal bone growth, endothelial cell growth, pericyte coverage, and bone formation by newly generated bone-forming cells occur in a precise spatial and temporal sequence, best visualized in metaphyseal growth plates. Growing endothelial tubes devoid of pericytes occupy the foremost 200 microns of the developing metaphysis [41]. Actively dividing abluminal pericytes and bone-forming osteoblasts are next in line. Progression of endochondral bone formation is dependent on efficient angiogenesis, and is blocked if angiogenesis is blocked, as illustrated by both experimental and pathological conditions. Experimentally, inhibition of VEGF signaling initiated by chondrocytes with blocking antibodies to the cognate receptor on growing blood vessels in the metaphysis results in a blockade not only of bone growth, but also of the related activities in the adjacent cartilage growth plates [42]. A remarkably similar event occurs naturally in rickets, and can be mimicked by microsurgical ablation of the metaphyseal vasculature [41].

Taking into account the similarities in their physical relationship to the vasculature, the cellular response to growth factors, and expression of similar markers lead one to suspect that marrow pericytes and marrow stromal cells are the same entity. Pericytes are perhaps one of the most elusive cell types in the body, and their significance as potential progenitor cells has been repeatedly surmised or postulated [28, 43-46]. Elegant as much as unconventional, experimental proof of their ability to generate cartilage and bone in vivo, for example, has been given in the past [47, 48]. Likewise, it has been shown that retinal pericytes form cartilage and bone (and express Stro-1) in vitro [49]. But, there has been little definitive understanding of the origin of this elusive cell type. Current evidence suggests that there is most likely more than one source of pericytes throughout development and growth. First, during development, pericytes may be recruited during angiogenesis or vasculogenesis from neighboring resident mesenchymal cells [50]. Secondly, as recently shown, pericytes may arise directly from endothelial cells or their progenitors [51, 52]. Third, they can be generated during

angiogenesis, either pre- or post-natally, through replication, migration and differentiation of other pericytes downstream of the growing vascular bud [32, 39, 53, 54]. With regards to bone marrow, this implies that marrow pericytes might also be heterogeneous in their mode of development and origin. Some may be recruited during blood vessel formation from resident, preexisting osteogenic cells; others may originate from endothelial cells; still others may grow from preexisting pericytes during vascular growth. Interestingly, it would be predicted from this model that a hierarchy of marrow stromal/progenitor cells exists. Some would be osteogenic in nature, while others would not. If so, one would expect to find multipotent cells with markers of osteogenic commitment, and multipotent cells with endothelial/pericytic markers. With respect to the phenotypic characterization of clonal stromal cells, evidence supporting a dual origin is indeed available.

ORTHODOX PLASTICITY OF MARROW STROMAL CELLS

As described above, stromal cells can take on many forms such as cartilage, bone, myelosupportive stroma, or fat. This behavior of marrow stromal cells, both in vitro and in vivo, has perhaps offered the first glimpse of the property now widely referred to as plasticity. It was shown, for example, that clonal strains of marrow adipocytes could be directed to an osteogenic differentiation and form genuine bone in an in vivo assay [55, 56]. Earlier, the ability of marrow reticular cells to convert to adipocytes in vivo had been noted [29, 57]. A number of different studies have claimed that fully differentiated chondrocytes can dedifferentiate in culture and then shift to an osteogenic phenotype [58, 59], and that similar or correlated events can be detected in vivo [60]. All of these data highlight the non-irreversible nature of the differentiation of several cell types otherwise seen as end points of various pathways/lineages (i.e., reticular cells, osteoblasts, chondrocytes, and adipocytes). The primary implication of these findings has remained largely unnoticed until recently. Commitment and differentiation are not usually thought of as reversible, but rather as multistep, unidirectional and terminal processes. This concept is reflected in the basic layout of virtually every scheme in every textbook depicting the organization of a multilineage system dependent on a stem cell. Here, a hierarchy of progenitors of progressively restricted differentiation potential is recognized or postulated. Lineages are segregated, leaving no room for switching phenotype at a “late” stage of differentiation, no way of turning red blood cells into white blood cells, for example. In contrast, it seems that one can turn an adipocyte or a chondrocyte into an osteoblast, and nature itself seems to do this under specific circumstances. If so, then some kind of reversible commitment is maintained until very late in the history of a single cell of the stromal system—a

notable and yet unnoticed singularity of the system, with broad biological significance.

There is a real physiological need for plasticity of connective tissue cells, namely the need to adapt different tissues that reside next to one another during organ growth, for example [30, 61], and it is likely that nature has evolved mechanisms for maintaining plasticity which remain to be fully elucidated. One example may be the key transcription factor controlling osteogenic commitment, *cbfa1* [62, 63], which is commonly if not constitutively expressed in stromal cells derived in culture from the post-natal marrow [12], and maintained during differentiation towards other “cell types” such as adipocytes. This is perhaps the most stringent proof that a cell “committed” to osteogenesis (as demonstrated by expression of the key gene of commitment) may still enter other pathways of differentiation that were thought to be alternative ones [61]. Whether one can isolate a multipotent *cbfa1*-negative (non-osteogenically committed) stromal cell is at present unclear. However, freshly isolated stromal cells sorted as *Stro-1^{bright}* have been shown to be *cbfa1*-negative by reverse transcriptase-polymerase chain reaction (Gronthos and Simmons, unpublished results). Interestingly, these cells also exhibit several endothelial markers, although never a true endothelial phenotype [21, 22].

The fact that chondrocytes, osteoblasts, reticular cells, and adipocytes come from a single precursor cell carrying a marker of osteogenic commitment is consistent with the fact that all of these cell types are members of the same organ, even though of different tissues. A single skeletal segment contains all of these cell types either at different stages of its own organogenesis or simultaneously. Although heretical to some and novel to others, even the notion that each of these cell phenotypes can switch to another within the same family under specific circumstances is consistent with the development and maintenance of the organ from which they were derived. This kind of plasticity is thus “orthodox,” meaning that it remains within the context of the organ system.

UNORTHODOX PLASTICITY OF MARROW STROMAL CELLS

Over the past 2 years, several studies have indicated or implied that progenitors can be found in a host of different post-natal tissues with the apparently “unorthodox” potential of differentiating into unrelated tissues. First, it was shown that the bone marrow contained systemically transplantable myogenic progenitors [64]. Second, it was shown that neural stem cells could reestablish hematopoiesis in irradiated mice [65]; third, that bone marrow cells could generate neural cells [66], and hepatocytes [67]; and fourth, that a neurogenic potential could be ascribed to marrow stromal cells [68, 69]. What is striking about these data is the developmentally

distant nature of the source of these progenitors and their ultimate destination. Differentiation across germ layers violates a consolidated law of developmental biology. Although consolidated laws are not dogmas (which elicited the comment that germ layers are more important to embryologists than to embryos), it is still indisputable and remarkable that even in embryos, cells with transgermal potential only exist under strict temporal and spatial constraints, with the notable exception of neural crest cells, which in spite of their neuroectodermal nature generate a number of craniofacial “mesodermal” tissues including bone. Cells grown in culture from the inner cell mass self-renew and maintain totipotency in culture for extended periods of time. However, this is in a way an artifact, of which we know some whys and wherefores (feeder cell layers, leukemia inhibitory factor). Embryonic stem (ES) cells only remain multipotent and self-renewing in the embryo itself for a very short period of time, after which totipotent cells only exist in the germline.

Consequently, the first key question is—where do the multipotent cells of post-natal organisms come from? All answers at this time are hypothetical at best. However, if marrow stromal cells are indeed members of a diffuse system of post-natal multipotent stem cells and they are at the same time vascular/pericytic in nature/origin, then a natural corollary would read that perhaps the microvasculature is a repository of multipotent cells in many, if not all, tissues [70]—a hypothesis that is currently being tested.

A second question is that if multipotent cells are everywhere, or almost everywhere, then what are the mechanisms by which differentiated cells keep their multipotency from making every organ a teratoma? Phrased in another way, adult tissues must retain some kind of organizing ability previously thought of as specific to embryonic organizers. If indeed cells in the bone marrow are able to become muscle or liver or brain, then there must be mechanisms ensuring that there is no liver or brain or muscle in the marrow. Hence, signals for maintenance of a tissue’s “self” must exist and be accomplished by differentiated cells. (That is, of course, if stem cells are not “differentiated” cells themselves).

A third question is—how much of the “stemness” (self-renewal and multipotency) observed in experimental systems is inherent to the cells that we manipulate, and how much is due to the manipulation? Are we discovering unknown and unexpected cells, or rather unknown and unexpected effects of manipulation of cells in culture? To what extent do cell culture conditions mimic the effects of an enucleated oocyte cytoplasm, which permits a somatic cell nucleus to generate an organism such as Dolly, the cloned sheep? For sure, a new definition of what a stem cell is—a timely, and biotechnologically correct, one—should incorporate the conditions

under which phenomena are recorded, rather than guessing from *ex vivo* performance what the true *in vivo* properties are. This exercise also has important implications for understanding where and when stem cells come into action in physiology. Even for the mother of all stem cells, the ES cell, self-renewal and multipotency are limited to specific times and events *in vivo*, and are much less limited *ex vivo*. Are similar constraints operating for other stem cells? Marrow stromal stem cells for example, can be expanded extensively in culture, but the majority of them likely never divide *in vivo* once skeletal growth has ceased (except the few that participate in bone turnover, and perhaps in response to injury or disease). What physiological mechanism calls for resumption of a “stem cell behavior” *in vivo* in the skeleton and other systems?

All of these questions are important not only for philosophical or esoteric reasons, but also for applicative purposes. Knowing even a few of the answers will undoubtedly enable biotechnology to better harness the magical properties of stem cells for clinical applications.

TRANSPLANTATION AND TRANSPLANTABILITY OF MARROW STROMAL CELLS

In vivo transplantation under defined experimental conditions has been the gold standard for defining the differentiation potential of marrow stromal cells, and a cardinal element of their very discovery. Historically, studies on the transplantability of marrow stromal cells are inscribed into the general problem of bone marrow transplantation (BMT). The HME is created by transplantation of marrow stromal cell strains and allows for the ectopic development of a hematopoietic tissue at the site of transplantation. The donor origin of the microenvironment and the host origin of hematopoiesis make the ectopic ossicle a true “reverse” BMT.

Local transplantation of marrow stromal cells for therapeutic applications permits the efficient reconstruction of bone defects larger than those that would spontaneously heal (critical size). A number of preclinical studies in animal models have convincingly shown the feasibility of marrow stromal cell grafts for orthopedic purposes [71-77], even though extensive work lies ahead in order to optimize the procedures, even in their simplest applications. For example, the ideal *ex vivo* expansion conditions have yet to be determined, or the composition and structure of the ideal carrier, or the numbers of cells that are required for regeneration of a volume of bone.

In addition to utilizing *ex vivo*-expanded BMSCs for regeneration of bone and associated tissues, evidence of the unorthodox plasticity of marrow stromal cells has suggested their potential use for unorthodox transplantation; that is, for example, to regenerate neural cells or deliver required gene

products at unorthodox sites such as the central nervous system (CNS) [78]. This could simplify an approach to cell therapy of the nervous system by eliminating the need for harvesting autologous human neural stem cells, an admittedly difficult procedure, although it is currently believed that heterologous cells may be used for the CNS, given the immune tolerance of the brain. Moreover, if indeed marrow stromal cells represent just a special case of post-natal multipotent stem cells, there is little doubt that they represent one of the most accessible sources of such cells for therapeutic use. The ease with which they are harvested (a simple marrow aspirate), and the simplicity of the procedures required for their culture and expansion *in vitro* may make them ideal candidates. For applicative purposes, understanding the actual differentiation spectrum of stromal stem cells requires further investigation. Besides neural cells, cardiomyocytes have been reported to represent another possible target of stromal cell manipulation and transplantation [79]. It also remains to be determined whether the myogenic progenitors found in the marrow [64] are indeed stromal (as some recent data would suggest, [80]) or non-stromal in nature [81], or both.

Given their residency in the marrow, and the prevailing view that marrow stromal cells fit into the hematopoietic paradigm, it was unavoidable that systemic transplantation of marrow stromal cells would be attempted [82] in order to cure more generalized skeletal diseases based on the successes of hematopoietic reconstitution by BMT. Yet major uncertainties remain in this area. Undoubtedly, the marrow stromal cell is the entity responsible for conveying genetic alterations into diseases of the skeleton. This is illustrated very well by the ability of these cells to recapitulate natural or targeted genetic abnormalities into abnormal bone formation in animal transplantation assays [83-85]. As such, they also represent a potential repository for therapy to alleviate bone disease. However, a significant rationale for the ability of stromal cells to colonize the skeleton once infused into the circulation is still missing.

The stroma is not transplanted along with hematopoiesis in standard BMT performed for hematological or oncological purposes [86-88]. Infusion of larger numbers of stromal cells than those present in cell preparations used for hematological BMT should be investigated further, as it might result, in principle, in limited engraftment. Stringent criteria must be adopted when assessing successful engraftment of systemically infused stromal cells [61]. The detection of reporter genes in tissue extracts or the isolation in culture of cells of donor origin does not prove cell engraftment; it proves cell survival. In this respect, it should be noted that even intra-arterial infusion of marrow stromal cells in a mouse limb may result in virtually no engraftment, even though abundant cells of donor origin are found impacted

within the marrow microvascular network. Of note, these non-engrafted cells would routinely be described as "engrafted" by the use of any reporter gene or *ex vivo* culture procedure. Less than stringent definitions of stromal cells (for example, their identification by generic or nonspecific markers) must be avoided when attempting their detection in the recipient's marrow. Clear-cut evidence for the sustained integration in the target tissue of differentiated cells of donor origin must be provided. This is rarely the case in current studies claiming engraftment of marrow stromal cells to the skeleton. Some evidence for a limited engraftment of skeletal progenitors following systemic infusion has, however, been obtained in animal models [89, 90]. These data match similar evidence for the possible delivery of marrow-derived myogenic progenitors to muscle via the systemic circulation [64]. It should be kept in mind that both skeletal and muscle tissues are normally formed during development and growth by extravascular cells that exploit migratory processes not involving the circulation. Is there an independent circulatory route for delivery of progenitors to solid phase tissues, and if so, are there physiologically circulating mesodermal progenitors? From where would these cells originate, both in development and post-natal organisms, and how would they negotiate the vessel wall? Addressing these questions is mandatory and requires extensive preclinical work.

Even once these issues are addressed, kinetic considerations regarding skeletal growth and turnover represent another major hurdle that must be overcome in order to cure systemic skeletal diseases via systemic infusion of skeletal progenitors. Yet there is broad opportunity for the treatment of single clinical episodes within the context of skeletal disease. While curing osteogenesis imperfecta by replacing the entire population of mutated skeletal progenitors with normal ones may remain an unattainable goal, individual fractures or deformity in osteogenesis imperfecta or FD of bone could be successfully treated with *ex vivo* "repaired" stromal cells, for example. Towards this end, future work must focus on the feasibility of transducing or otherwise genetically correcting autologous mutated osteoprogenitors *ex vivo*, and studies are beginning to move in this direction.

GENE TRANSFER AND THERAPY UTILIZING BMSCs

Molecular engineering of cells, either transiently or permanently, has become a mainstay in cell and molecular biology, leading to many exciting insights into the role of a given protein in cell metabolism both *in vitro* and *in vivo*. Application of these techniques for correcting human deficiencies and disease is a challenge that is currently receiving much attention. BMSCs offer a unique opportunity to establish transplantation schemes to correct genetic diseases of the skeleton. They may be easily obtained from the future

recipient, manipulated genetically and expanded in number before reintroduction. This eliminates not only the complications of xenografts, but also bypasses the limitations and risks connected with delivery of genetic repair material directly to the patient via pathogen-associated vectors. While a similar strategy may be applied to ES cells, the use of post-natal BMSCs is preferable considering that they can be used autologously, thereby avoiding possible immunological complications from a xenograft. Furthermore, there is far less concern of inappropriate differentiation as may occur with ES cell transplantation. Finally, ES cell transplantation is highly controversial, and it is likely that the ethical debate surrounding their usage will continue for quite some time.

Depending on the situation, there are several approaches that can be envisioned. If a short-lived effect is the goal, such as in speeding up bone regeneration, transient transduction would be the desired outcome, utilizing methods such as electroporation, chemical methods including calcium phosphate precipitation and lipofection, and plasmids and viral constructs such as adenovirus. Transducing BMSCs with adenoviral constructs containing BMP-2 has demonstrated at least partial efficacy of this approach in hastening bone regeneration in animal models [75, 91, 92]. Adenoviral techniques are attractive due to the lack of toxicity; however, the level at which BMSCs are transfected is variable, and problematic. It has been reported that normal, non-transformed BMSCs require 10× more infective agent than other cell types [93], which is often associated with cellular toxicity. Clearly, further optimization is needed for full implementation of this approach.

For treatment of recessive diseases in which a biological activity is either missing or diminished, long-lasting or permanent transduction is required, and has depended on the use of adeno-associated viruses, retroviruses, lentiviruses (a subclass of retrovirus), and more recently, adeno-retroviral chimeras [94]. These viruses are able to accommodate large constructs of DNA (up to 8 kb), and while retroviruses require active proliferation for efficient transfection, lentiviruses do not. Exogenous biological activity in BMSCs by transduction with retroviral constructs directing the synthesis of reporter molecules, interleukin 3, CD-2, Factor VIII, or the enzymes that synthesize L-DOPA has been reported [78, 95-102]. However, these studies also highlight some of the hurdles that must be overcome before this technology will become practical. The first hurdle is optimization of ex vivo transfection. It has been reported that lengthy ex vivo expansion (3-4 weeks) to increase cell numbers reduces transfectability of BMSCs, whereas short-term culture (10-12 days) does not [98]. Furthermore, high levels of transduction may require multiple rounds of transfection [95, 101]. The second hurdle relates to

the durability of the desired gene expression. No reported study has extended beyond 4 months post-transplantation of transduced cells [99] (*Gronthos*, unpublished results), and in most instances, it has been reported that expression decreases with time [96], due to promoter inactivation [102] and/or loss of transduced cells (*Mankani* and *Robey*, unpublished results). While promising, these results point to the need for careful consideration of the ex vivo methods, choice of promoter to drive the desired biological activity, and assessment of the ability of the transduced BMSCs to retain their ability to self-maintain upon in vivo transplantation. It must also be pointed out that using retrovirally transduced BMSCs for this type of application, providing a missing or decreased biological activity, does not necessarily require that they truly engraft, as defined above. They may be able to perform this function by remaining resident without actually physically incorporating and functioning within a connective tissue. In this case, they can be envisioned as forming an in vivo biological mini-pump as a means of introducing a required factor, as opposed to standard means of oral or systemic administration.

Use of transduced BMSCs for the treatment of a dominant negative disease, in which there is actual expression of malfunctioning or inappropriate biological activity, is far more problematic, independent of whether we are able to deliver BMSCs systemically or orthotopically. In this case, an activity must be silenced such that it does not interfere with any normal activity that is present, or reintroduced by any other means. The most direct approach would be the application of homologous recombination, as applied to ES cells and generation of transgenic animals. The almost vanishing low rate of homologous recombination in current methodology, coupled with issues of the identification, separation, and expansion of such recombinants does not make this seem feasible in the near future. However, new techniques for increasing the rate of homologous recombinations are under development [103] which may make this approach more feasible. Another approach to gene therapy is based on the processes whereby mismatches in DNA heteroduplexes that arise sporadically during normal cell activity are automatically corrected. Genetic mutations could be targeted by introducing exogenous DNA with the desired sequence (either short DNA oligonucleotides or chimeric RNA/DNA oligonucleotides) which binds to homologous sequences in the genome forming a heteroduplex that is then rectified by a number of naturally occurring repair processes [104]. A third option exists using a specially constructed oligonucleotide that binds to the gene in question to form a triple helical structure, thereby disallowing gene transcription [105].

While it would be highly desirable to correct a genetic disease at the genomic level, mRNA represents another very significant target, and perhaps a more accessible one, to silence the

activity of a dominant negative gene. Methods for inhibiting mRNA translation and/or increasing its degradation have been employed through the use of protein decoys to prevent association of a particular mRNA to the biosynthetic machinery and antisense sequences (either oligonucleotides or full-length sequences). Double-stranded RNA also induces rapid degradation of mRNA (termed RNA interference, RNAi) by a process that is not well understood [105]. However, eliminating mRNAs transcribed from a mutant allele with short or single-base mutations by these approaches would most likely not maintain mRNA from a normal allele. For this reason, hammerhead and hairpin ribozymes represent yet another alternative, based on their ability to bind to very specific sequences, and to cleave them and inactivate them from subsequent translation. Consequently, incorporating a mutant sequence, even one that transcribes a single base mutation, can direct a hammerhead or hairpin ribozyme to inactivate a very specific mRNA. This approach is currently being probed for its possible use in the treatment of osteogenesis imperfecta [106]. Taking this technology one step further, DNazymes that mimic the enzymatic activity of ribozymes, which would be far more stable than ribozymes, are also being developed. Regardless of whether genomic or cytoplasmic sequences are the target of gene therapy, the efficacy of all of these new technologies will depend on: A) the efficiency at which the reagents are

incorporated into BMSCs in the ex vivo environment; B) the selection of specific targets, and C) the maintenance of the ability of BMSCs to function appropriately in vitro.

In conclusion, the isolation of post-natal stem cells from a variety of tissues along with discovery of their unexpected capabilities has provided us with a new conceptual framework in which to both view them and use them. However, even with this new perspective, there is much to be done to better understand them: their origins, their relationships to one another, their ability to differentiate or re-differentiate, their physiological role during development, growth, and maturity, and in disease. These types of studies will most certainly require a great deal of interdisciplinary crosstalk between investigators in the areas of natal and post-natal development, and in different organ systems. Clearly, as these studies progress, open mindedness will be needed to better understand the nature of this exciting family of cells, as well as to better understand the full utilization of stem cells with or without genetic manipulation. Much to be learned. Much to be gained.

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