

Plasticity of Bone Marrow–Derived Stem Cells

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ABSTRACT

Stem cell plasticity refers to the ability of adult stem cells to acquire mature phenotypes that are different from their tissue of origin. Adult bone marrow cells (BMCs) include two populations of bone marrow stem cells (BMCs): hematopoietic stem cells (HSCs), which give rise to all mature lineages of blood, and mesenchymal stem cells (MSCs), which can differentiate into bone, cartilage, and fat. In this article, we review the literature that lends

credibility to the theory that highly plastic BMCs have a role in maintenance and repair of nonhematopoietic tissue. We discuss the possible mechanisms by which this may occur. Also reviewed is the possibility that adult BMCs can change their gene expression profile after fusion with a mature cell, which has brought into question whether this stem cell plasticity is real. *Stem Cells* 2004; 22:487–500

STEM CELLS IN ADULT BONE MARROW (BM)

The main function of stem cells in adult tissue is to repair and regenerate the tissue in which they reside. Stem cells have the ability to self-renew and to differentiate into at least one mature cell type. Under normal conditions, stem cells divide to produce progenitor cells that can, depending on the tissue, go through a number of subsequent cell divisions and differentiation steps to produce a complex web of mature cells. Hematopoietic stem cells (HSCs) are a well-characterized population of self-renewing cells that produce progenitors that differentiate into every type of mature blood cell in a well-defined hierarchy [1].

Mesenchymal stem cells (MSCs), which reside in bone marrow as well as in other tissues (e.g., fat), adhere to plastic in vitro and expand in tissue culture with a finite lifespan of 15–50 cell doublings. Under appropriate stimuli, MSCs differentiate in vitro and in vivo into adipocytes, chondrocytes, and osteoblasts [2, 3]. Although MSCs are stem cells, capable of self-renewal and multilineage differentiation, it is not yet clear to what extent MSCs are responsible for normal growth or maintenance in vivo.

A relatively elusive adherent stem-cell population referred to as multipotent adult progenitor cells (MAPCs) can be isolated by in vitro growth of bone marrow cells (BMCs) in growth medium containing specific growth factors (e.g., epidermal growth factor and platelet-derived growth factor) for several months while maintaining a relatively low cell density of $0.5\text{--}1.5 \times 10^3$ cells/cm². MAPCs have the ability to form classical endodermal, mesodermal, and ectodermally derived cell types such as hepatocytes, endothelial cells, and neurons in vitro [4]. The pluripotentiality of MAPCs has been confirmed in vivo; MAPCs can contribute to multiple tissues (including brain, retina, lung, myocardium, skeletal, muscle, intestine, kidney, spleen, bone marrow, blood, and skin) in chimeric mice that are derived from injection of MAPCs into early blastocysts. Also, MAPCs can engraft hematopoietic tissues (blood, bone marrow, and spleen), as well as mature epithelial cells of the lung, liver, and intestine when administered intravenously to sublethally irradiated adult nonobese diabetic/ severe combined immunodeficient (NOD/SCID) mice [4]. Unlike hematopoietic cells, undifferentiated MAPCs do not express CD45, c-Kit, or Sca-1. These cells

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may represent a rare subpopulation of MSCs, or they may be a byproduct of the long-term in vitro culture conditions used for their purification.

EVIDENCE FOR BONE MARROW STEM CELL (BMC) PLASTICITY

BMCs to Skeletal Muscle

Muscle regeneration and repair are thought to be carried out by muscle-specific stem cells, called satellite cells, which are located between the myocytes and the basal lamina of skeletal muscle fibers [5–7]. Characteristic of stem cells, satellite cells are usually quiescent and have the ability to both self-renew and to generate new skeletal muscle fibers. Derivation of skeletal muscle myocytes from BMCs has been studied in vivo after muscle injury and in mouse models of degenerative muscle disease.

The first in vivo evidence of BM-derived cells contributing to muscle regeneration came from a study using BMCs from C57/BLacZ transgenic mice that express the β -galactosidase (β -gal) reporter gene under the muscle-specific myosin light chain promoter [8]. Unfractionated, adherent, or nonadherent BMCs were injected directly into the tibialis anterior muscle of immunodeficient mice 24 hours after damage was induced with cardiotoxin injection. As a control, each animal was injected with 10^5 purified satellite cells into a similarly damaged contralateral muscle. After 2–5 weeks, four of six mice injected with unfractionated BMCs had β -gal⁺ nuclei within their skeletal muscle fibers. In their contralateral control leg, each animal had muscle fibers that had incorporated the satellite cells at a higher level of engraftment than that observed for BMCs [8]. The BMCs did not need to be injected directly into the muscle; after bone marrow transplantation (BMT), intravenously injected cells also differentiate into muscle cells in vivo. The location of the β -gal⁺ nuclei within the muscle fibers of transplant recipients suggested that both immature and mature muscle fibers developed from the BM donor cells [8].

Evidence that BMCs might be recruited to differentiate into skeletal muscle fibers under physiologic conditions when the local satellite cell population is depleted was obtained in mouse models for Duchenne muscular dystrophy, *mdx* and *mdx4cv* transgenic mice [9–11]. These mice lack functional dystrophin. Early in the disease, the muscle mass is maintained by resident satellite cells; however, over time, the muscle is replaced by connective tissue, thereby leading to fibrosis and muscular dystrophy [9]. Ten weeks after BMT, *mdx* mice transplanted with normal BM had donor-derived cells that costained for muscle-specific markers myogenin and myf-5, and some of these BM-derived muscle cells expressed dystrophin [9].

Side-population (SP) BMCs, characterized by the ability to extrude Hoechst dye, and to fluoresce with a unique pattern on fluorescence-activated cell sorter (FACS) analysis, can also become incorporated into skeletal muscle fibers. SP cells are CD45⁺, CD34^{-/low}, c-Kit^{+/dim}, Sca-1⁺ and enriched for HSCs [12–14]. A potential problem with the *mdx* mouse model is that a background of muscle fibers may spontaneously revert to synthesize normal dystrophin, so that the detection of dystrophin alone is not conclusive evidence that BMCs differentiate into functional muscle fibers. Twelve weeks post-BMT with SP cells, dystrophin is expressed in up to 4% of myofibers, and of these dystrophin-expressing fibers, donor-derived nuclei were detected in 10%–30% [10]. BMCs were also transplanted into *mdx4cv* transgenic mice that exhibit an extremely low percentage (0.2%) of reverting fibers. Similar to previous experiments, BMCs demonstrated myogenic potential by engrafting into the skeletal muscles of mice with muscular dystrophy. Normal dystrophin was expressed in these animals, suggesting that the BMC-derived myoblasts were functional; however, the level of dystrophin-positive fibers averaged only 0.25% throughout the 10-month period of analysis and never exceeded 1% [15].

In a rigorous study tracking the pathway of BMCs to satellite cell to myofiber, unfractionated green fluorescent protein-positive (GFP⁺) BMCs were transplanted into irradiated recipients [16]. Irradiation served to both ablate the BM compartment and decrease satellite stem cell numbers in muscle tissue. GFP⁺, BMC-derived satellite cells were identified in muscle tissue of BMT recipients by morphology and also by their ability to self-renew and differentiate into myotubes in vitro. The cells were karyotyped, and the “re-programmed” cells were diploid. The level of BMC-derived multinucleate muscle fibers in BMT-recipient mice was greatly increased when the animals underwent physical activity for 6 months [16]. This study is important because it provides evidence that BM to muscle differentiation occurs via repopulation of the muscle stem cell compartment. Although it is not yet known which BM subpopulations have the ability to develop into muscle, the data obtained using SP cells suggest that a population enriched for HSCs is responsible [10]. Muscle damage may not be a prerequisite for differentiation from BM to skeletal muscle. For example, 16 months post-BMT, recipient mice had engraftment of BMC into the panniculus carnosus muscle, which is much higher than what was seen in any other muscle analyzed, suggesting that BMCs may contribute to regeneration of the panniculus carnosus under normal physiologic conditions [17, 18].

Although the studies described above provide evidence that differentiation from BM to muscle occurs via repopula-

tion of the muscle stem cell compartment, some of the incorporation of marrow-derived cells into skeletal muscle may be due to fusion. Work by Ye et al. [19], using a Cre-Lox system as a readout for cell–cell fusion, supports this theory. They used, as recipients of BMT, mice that have a Stop-Lox- β -gal cassette, which allows for expression of β -gal by a cell only if the nonmammalian Cre recombinase enzyme is expressed. As the BM donors, they used transgenic mice that express Cre from the lysozyme M promoter, which is active predominantly, although not exclusively [19], in myeloid cells. After BMT, recipients had β -gal-expressing muscle fibers. Although these data strongly suggest that the β -gal⁺ muscle may have developed by fusion of a donor-derived myeloid cell with a pre-existing muscle fiber, they are not conclusive because complete cell-type specificity of the Lys-M promoter is lacking [19].

BMCs to Cardiac Muscle

BMCs can also incorporate into cardiac muscle fibers. Data showing differentiation of cardiac myocytes in vitro and in vivo suggest that cell therapy may be effective for cardiac disorders such as acute myocardial infarction [20], chronic ischemia [21, 22], and cardiac graft rejection [23]. In vivo, BM-derived stromal cells and mesenchymal stem cells can differentiate into cardiac myocytes, as assessed by morphology, spontaneous beating, or muscle-specific protein expression [24, 25]. One of the manipulations that induces this differentiation is addition of 5-azacytidine to the growth medium. This drug leads to DNA demethylation and may thereby activate previously silenced regions of the genomic DNA.

Myocardial infarction in humans and in animal models by ligation of the left main coronary artery leads to death of myocytes and vascular structures in the affected area. Left untreated, the remaining myocytes do not reconstitute the injured tissue, and scarring occurs, which can lead to deterioration of cardiac function [26–28]. Injection of lineage-depleted, c-Kit⁺ BMC-derived hematopoietic progenitor cells directly into intact myocardium bordering the infarct area can promote regeneration of functional myocardium [20]. BMCs migrate into the necrotic area of the infarcted myocardium and regenerate myocytes, as well as vascular structures. Even though the thinning of the infarcted heart is only partially restored, BM-derived myocytes appear to be able to restore electrical connections, leading to a functional improvement of ventricular activity.

HSCs can be mobilized from BM to the peripheral blood by administration of hematopoietic growth factors. When stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) were used to mobilize HSCs 5 days before and 3 days after ligation of the left coronary artery in mice,

there was significantly less mortality, improved cardiac function, and more tissue repair with proliferating myocytes and newly formed vessels than in the control animals that had not received growth factors [20]. Based on these data, it is tempting to speculate that mobilized BMCs may respond to coronary occlusion by migrating to the site of injury and generating new myocardium. However, this study was not designed to assess whether the circulating BM-derived cells directly contributed to the improved cardiac outcome. Consistent with these findings, among patients who have had acute myocardial infarction, those in whom immature hematopoietic cells are mobilized from the bone marrow show a statistically significant improvement in ejection fraction 6 months after myocardial infarction than similar patients in whom no mobilization of hematopoietic progenitors occurred [29].

Engrafted BM SP cells can also contribute to myocardial tissue. When BMT recipients of β -gal⁺ BM SP cells underwent occlusion and reperfusion of the left descending coronary artery, BMC had contributed to regenerating heart tissue as both endothelial cells and myocytes. These β -gal⁺ cells costained for α -actinin and seemed to be connected to host myocytes [13].

Stromal cells injected into scarred ventricles 3 weeks after myocardial infarction can also engraft in the wounded tissue and express troponin 1 and myosin heavy chain and can increase cardiac function significantly [24]. In a similar in vivo experiment, rat bone marrow stromal cell-derived cardiac myocytes expressed contractile proteins and connexin 43 [30]. This gap junction protein was seen between BMC-derived myocytes and host myocytes, suggesting the donor-derived cells integrated themselves into the cardiac muscle network that was necessary for electrophysiological function [30].

BMC-derived cardiac myocytes also may have therapeutic potential for the treatment of chronic cardiac graft rejection after heart transplantation. Immunologic graft rejection compromises cardiac function due to fibrosis and scarring [31, 32]. MSCs home to and engraft within allogeneic cardiac grafts that are undergoing graft rejection. IV injection into rats of β -gal⁺ rat MSCs 1 week before and 1, 2, and 3 weeks after allogeneic heart transplantation resulted in localization of the MSCs to fibrotic regions of the graft and significantly reduce graft survival time. Although most of the MSC-derived cells were fibroblastic in appearance, a small proportion of the MSC-derived cells expressed desmin, which is unique to cardiac myocytes in the heart [33].

To assess the ability of human BMCs to incorporate into cardiac tissue, CD34-selected cells from mobilized human peripheral blood were injected into rats 48 hours after ligation of the left anterior descending coronary artery [34]. Two weeks post-transplantation, animals that had received human

cells had an increase in vascularity and cellularity within the area of infarct, as well as decreased fibrosis, over that of the sham-operated controls. Moreover, using echocardiography, cardiac output, and ventricular volume measurements, cardiac function had improved in rats that received these CD34⁺ cells following ischemic injury [34].

BM-derived cells may also differentiate into cardiac myocytes in humans. In a retrospective study of heart transplant recipients, the heart tissue of eight males who had received female hearts showed high levels of chimerism; up to 18% of myocytes, 20% of coronary arterioles, and 14% of capillaries within the allogeneic hearts were Y-chromosome positive [35]. The kinetics of this engraftment are not yet known, nor is it known whether BM engraftment into cardiac tissue is a result of fusion with endogenous cells. The relatively high level of engraftment is likely related to cardiac injury secondary to rejection because the hearts of female patients who had undergone BMT from male donors had an average of only 0.23% Y positive, α -sarcomeric actin-positive cardiac myocytes [36]. The normal karyotype of these cells suggested that fusion had not occurred. The relatively low percentage of BMC-derived myocytes may reflect the degree to which BMCs contribute to heart tissue under physiological conditions [36].

Clinical studies investigating the effect of injecting autologous BMCs into humans with chronic myocardial ischemia show that intramyocardial injection of BM-derived cells is safe and may improve outcome in treated patients. For example, Perin et al. [22] evaluated 14 patients with chronic cardiac ischemia who had been treated with direct intracardiac injections of approximately 25 million mononuclear BMCs per patient. Cells were injected into ischemic areas containing viable myocardium, which the researchers believed was necessary to support neovascularization. After 4 months, the patients had improved cardiac perfusion and contractility compared with their baseline measurements. It is not known whether the injected cells facilitated repair in myocardial tissue directly (by engrafting as cardiac myocytes or endothelial cells) or indirectly (by stimulating angiogenesis or myogenesis). In a related clinical trial, autologous skeletal muscle cell transplantation into patients with heart disease undergoing left ventricular assist device implantation proved to be safe [37].

As of January 2004, data from several clinical trials in which marrow-derived cells have been injected into myocardial tissue in humans have been reported [21, 22, 38–42]. No short-term toxicity or adverse events have been reported. Although limitations of each of the studies have hindered our ability to conclude whether a therapeutic benefit of administering marrow-derived cells has occurred, the data are promising [43].

BMCs to Liver

Liver has at least two internal mechanisms for repair and maintenance. The primary mechanism of liver generation and repair is proliferation and hypertrophy of existing hepatocytes and cholangiocytes. When liver is severely damaged and endogenous hepatocytes cannot divide, then liver repair is facilitated by oval cells, which act as tissue-specific stem cells. It is generally agreed that oval cells, located around bile ducts with oval-shaped nuclei, comprise a resident bipotent liver stem cell population [44]. Oval cells proliferate and differentiate in rats after hepatic injury with carbon tetrachloride (CCl₄) when endogenous hepatocyte proliferation is suppressed by 2-acetylaminofluorene (2-AAF) [23]. When BMT using unfractionated bone marrow from dipeptidyl peptidase IV-positive (DDPIV⁺) rats into DDPIV-null (DDPIV⁻) rats was performed, followed by liver damage with 2-AAF and CCl₄ 2 weeks later, DDPIV⁺ donor-derived oval cells and hepatocytes developed [23]. BMC-derived hepatocytes ranged from 0.76%–2.2% of total hepatocytes. In humans, sex-mismatched BMT recipients have BM-derived hepatocytes and cholangiocytes, suggesting that donor-derived marrow cells may have engrafted as oval cells [45, 46].

Results from xenotransplantation of human BMCs into sublethally irradiated immunodeficient mice suggest that HSC-enriched populations contain cells that engraft as mature hepatocytes in the liver. Lin⁻CD38⁻CD34⁺ClqR⁺ cells contribute to hematopoiesis in sublethally irradiated NOD/SCID mice and engraft as mature human hepatocytes [47]. In a related xenotransplantation study, human hepatocytes developed in the livers of mice that had been injected with either human CD34⁺ BMCs or CD34⁺ cord blood cells and treated with the hepatotoxin CCl₄ 1 month post-transplantation [48]. The level of human albumin RNA expressed in transplanted, injured mice increased significantly with human hepatocyte growth factor treatment [48].

To determine whether a single marrow-derived cell can differentiate into both hematopoietic and epithelial cell types, Krause et al. [49] performed transplanted single male-derived BMCs into lethally irradiated female mice. The HSC population used was isolated by a multistep process. First, the male mouse BMCs were purified by elutriation, and the lineage was depleted to enrich for long-term repopulating cells. These cells were then labeled with PKH26, a membrane dye, and then transplanted into primary lethally irradiated female hosts. After 48 hours, the PKH26-labeled cells were recovered from the bone marrow, and single cells were transplanted into lethally irradiated secondary female recipients using limiting dilution. In the mice that survived the single-cell transplantation, there was male-derived hematopoietic reconstitution, and all of the mice had Y chromosome-positive epithelial cells in the lung, liver, skin, and

gastrointestinal (GI) tract [49]. Another murine transplantation study demonstrated that a single, long-term, bone marrow–repopulating c-Kit⁺, Thy1.1¹⁰, Lin⁻, Sca-1⁺ (KTLS) cell could, in a rare event, incorporate into the liver as hepatocytes and into the central nervous system as Purkinje cells [50].

Functionality of BM-derived hepatocytes was demonstrated in a mouse model for the metabolic liver disease tyrosinemia type I [51]. These mice are deficient in the enzyme fumarylacetoacetate hydrolase (FAH) and must be maintained on a drug, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) to survive. FAH-deficient mice that had undergone BMT using wild-type (FAH⁺) donors were taken off NTBC 3 weeks post-transplantation, and four of nine mice survived with substantially improved liver function. Livers of these mice had large segments containing functional FAH⁺ hepatocytes that were derived from the BM donor. The dramatic survival advantage of the marrow-derived hepatocytes in this liver disease model allowed BM-derived hepatocytes to expand and reconstitute up to 50% of the recipient liver. In this model system, data suggest that the mechanism by which the BM-derived cells changed their gene expression pattern to that of mature functional hepatocytes is likely to be fusion with damaged recipient liver cells [52, 53].

Kanazawa and Verma [54] tested the ability of BMCs to generate hepatocytes in other mouse models of liver disease: an albumin-urokinase transgenic mouse and a hepatitis B transgenic mouse. When these animals underwent lethal irradiation and transplantation with bone marrow from GFP or β -gal transgenic mice, no GFP⁺ or β -gal⁺ hepatocytes were found. However, when the BMT recipient mice were treated with the hepatotoxin CCl₄, then donor-derived hepatocytes (as assessed by Y chromosome fluorescence in situ hybridization [FISH] methodology) were identified in their liver tissue showing that BMC-to-liver-cell plasticity did occur at a frequency of approximately 1 in 8×10^4 hepatocytes [54]. This paper highlights a problem that has been picked up by others: Transgene expression is a less consistent means of detecting donor-derived cells than is Y chromosome in situ hybridization, and neither of the disease models was analyzed with Y chromosome in situ hybridization. We need to take this into account when analyzing published results based on GFP or β -gal expression.

MAPCs, derived from mouse, rat, and human bone marrow can differentiate into functional hepatocyte cells in vitro [55]. Optimal conditions for in vitro differentiation of MAPC to liver were achieved by plating the cells at a density of 2×10^4 cells per cm² on a combination of extracellular matrix proteins (matrigel) in the presence of fibroblast growth factor-4 (FGF-4) and human growth factor (HGF). After 2 weeks the

cells were characterized as hepatocyte-like cells, expressing early and late markers of hepatocytes. The cells also had functional characteristics of hepatocytes, including the production and secretion of urea and albumin, cytochrome P450 activity, and uptake of low-density lipoprotein.

BMCs to Skin

Adult skin contains multipotent tissue stem cells that can self-renew and give rise to epidermis, sebaceous glands, and hair follicles [56]. This putative stem cell resides in the bulge region of hair follicles [57]. Long-term repopulating BMCs can differentiate into cytokeratin-positive epithelium of the epidermis [49]. Using a BMT model, BMC-derived cells were found in the bulge region of the follicle, yet they did not show clonal expansion. In humans, female recipients of male BMTs had BMC engraftment into the epidermis (as well as the GI tract and liver), as determined by colocalization of the donor Y chromosome and cytokeratin in recipient tissue [58]. In a separate study, skin biopsies from females who had undergone transplantation with male peripheral blood stem cells were assessed not only for the presence of Y-positive epithelial cells but also for skin stem cells as assessed by self-renewal in vitro. Consistent with other reports, epidermal tissue from the patients had cytokeratin-positive, Y-positive cells, suggesting that they were BM-derived keratinocytes. The degree of engraftment of marrow-derived keratinocytes did not correlate with the degree of graft-versus-host disease (GVHD) in the skin ([59], J. Grove, unpublished data). No donor-derived keratinocytes grew in vitro, suggesting that the donor-derived cells detected in vivo could not survive under the in vitro culture conditions used [59].

BMCs to GI Tract

The GI tract of mammals is thought to have a resident population of tissue stem cells that contribute to the continual turnover of the gut epithelium. The putative multipotent intestinal stem cells reside in the crypts and give rise to all types of epithelial cells, columnar absorptive enterocytes, Paneth cells, goblet cells, and enteroendocrine cells [60–62]. BM-derived intestinal epithelial cells were first shown by Krause et al. [49], in the single-cell study described above. Since then, data provided by other studies have corroborated this finding. In one study, four female cancer patients receiving sex-mismatched BMT were chosen for analysis because they displayed symptoms of chronic GVHD, acute GVHD, peptic ulcer, or inflammation of the GI tract. Donor-derived epithelial cells were identified in the GI tract of each of the BMT recipients [63]. The male donor cells were found in all sections of the GI tract, including the esophagus, stomach, small intestine, and colon, and they appeared to increase during

episodes of GVHD. This is in contrast to findings in the human skin in which the percentage of BM-derived keratinocytes was not affected by the GVHD status of the patient [58, 59]. Chromosomal karyotyping ruled out the possibility that Y-positive, cytokeratin-positive cells were a result of stable fusion [63]. Donor-derived, cytokeratin-positive cells were found in the GI tract of two female cancer patients who received CD34⁺-selected peripheral blood transplants from brothers following a myeloablative treatment [58]. While these patients also had episodes of GVHD, no correlation was found in these two patients between GVHD and BMC engraftment. Taken together, these studies support the theory that a BMC population enriched for HSC contains cells that can differentiate into intestinal epithelial cells.

BMCs also differentiate into intestinal myofibroblasts in mice and humans [64]. The GI tract of female mice that had been transplanted with male BMCs were analyzed 7, 14, and 42 days post-transplantation by FISH for the Y chromosome and immunohistochemistry for smooth muscle actin (SMA), desmin, F4/80 (macrophages), and CD34 (hematopoietic progenitors). Around the crypt region, donor-derived myofibroblasts (SMA⁺, desmin⁻, F4/80⁻, CD34⁻ cells) were seen in BMT recipients after 7 days; by 14 days, 49.4% of myofibroblasts around the crypt were marrow-derived, and after 6 weeks, 57.6% were Y-chromosome positive [64]. Similarly in women who had undergone BMT, Y-positive, SMA-positive pericryptal myofibroblasts were found in three of three patients studied [64]. The fact that BMCs differentiate into myofibroblast cells following BMT suggests that BM-derived cells may have a supportive role in the regeneration of intestinal epithelium by providing soluble factors, cytokines, and the necessary milieu to produce new tissue.

BMCs to Lung

Type II pneumocytes are the putative lung stem cells responsible for alveolar tissue turnover. In response to injury, these cells proliferate to create more type II cells and also differentiate into type I pneumocytes [65]. BMCs capable of long-term hematopoietic reconstitution can become type II pneumocytes following BMT [49]. BM-derived type II pneumocytes have been identified by colocalization of the Y chromosome and cytokeratin, as well as by expression of surfactant protein B mRNA, which is specific to type II pneumocytes in the alveoli [49]. After BMT, the level of engraftment of donor-derived epithelial cells is greater in the lung than in other epithelial tissues, perhaps because of radiation-induced pneumonitis [66]. Following lethal irradiation and BMT with male BM, BM-derived cells begin to repopulate the damaged alveolar tissue after 5–7 days, and clusters of BM-derived epithelial cells are present by 2 months and thereafter. The percentage of donor-derived cells that differentiate into type II pneumocytes ranges from 0.7% at day 5 to 14% at

6 months [66]. BM-derived type II pneumocytes also developed in mice transplanted with CD34⁺Lin⁻ male BM and analyzed 8 months post-transplantation, confirming that a BM subpopulation enriched for hematopoietic progenitors can engraft in irradiated lung tissue as pneumocytes.

Marrow stromal cells may also be able to incorporate into epithelial tissue in the lung. Kotton et al. [67] used a bleomycin injury protocol to damage the alveolar tissue of mice before injecting plastic-adherent BMCs that had been cultured *in vitro* for 10 days. The injected cells formed clusters of type I pneumocytes *in vivo* after 30 days, and analysis at shorter time points suggested that BMCs differentiate directly into type I pneumocytes rather than first engrafting as type II cells. Because the marrow-derived cell population was not depleted of CD45⁺ hematopoietic cells, it cannot be determined whether MSCs were responsible for the observed engraftment of pneumocytes. More recently, a group performed a similar study using purified MSCs. The MSCs were isolated from bleomycin-resistant male mice by plastic adherence, and then hematopoietic progenitors were removed by immunodepletion. These cells were injected intravenously into bleomycin-sensitive female mice that had been exposed to bleomycin. Type II pneumocytes were isolated and analyzed by real-time polymerase chain reaction (PCR) and FISH for Y chromosome. Both methods of analysis suggested the presence of male, BM-derived type II pneumocytes [68].

In a small study of gender-mismatched human lung transplantation, 6%–26% of bronchial epithelial cells, 9%–20% of pneumocytes, and 9%–24% of seromucous glands were recipient-derived [69]. The investigators developed a microdissection and PCR technique to detect donor-derived cells, which resulted in data comparable to those obtained using Y-chromosome *in situ* hybridization. A correlation between the level of recipient engraftment and the degree of chronic injury was noted, which supports other studies finding increasing numbers of BM-derived cells in the lungs of animals who had undergone bleomycin injury [67, 68]. It cannot be determined whether the recipient-derived lung cells in the transplanted lungs were from bone marrow. Again, consistent with tissue damage facilitating the transition from BM to epithelial cell, no donor-derived lung cells were detected in BMT recipients [69]. This is a problem because the authors regarded this experimental group as a good negative control for the microdissection and PCR technique. These data differ from those of Suratt et al. [108], who showed that two of three female recipients of BMT using male donor cells had a 2.5%–8% epithelial donor chimerism in biopsied lung tissue as detected by Y-chromosome FISH. It is likely that tissue injury, while not sufficient, is necessary for engraftment of marrow-derived cells as epithelial cells. This is a critical discovery, as it may limit the therapeutic

potential of using marrow-derived cells to treat diseases in which tissue injury does not occur. For example, in the absence of tissue injury, no engraftment of nasal epithelial cells is detectable in female cystic fibrosis patients who, for treatment of malignancy, had undergone BMT with male donors [70]. To date, there is no evidence that BMCs can perform the normal functions of lung epithelial cells.

BMCs to Pancreas

BMCs can also differentiate into pancreatic endocrine β cells [71, 72]. For these studies, bone marrow donors were double-transgenic mice that express Cre recombinase on an insulin promoter (INS2-CRE) and contain three translational stop codons flanked by Lox P sites upstream of enhanced green fluorescent protein (GFP) on the ROSA26 locus promoter (ROSA-stoplox-GFP) [71]. When the insulin promoter is active in cells from these mice, Cre recombinase excises the stop codons so that GFP is expressed. BMCs from male transgenic mice were transplanted into lethally irradiated wild-type recipients, and the mice that survived had Y chromosome-positive peripheral blood cells. Moreover, GFP-positive cells were found in the islets; these cells expressed insulin and transcription factors specific for β cells. To test whether BMCs were incorporated as pancreatic β cells by fusion, a sex-mismatched Cre-Lox approach was used. BMT was performed using BM from single-transgenic male mice that express Cre recombinase on the insulin II promoter. The recipients were lethally irradiated female ROSA-stoplox-GFP recipients. In this situation, GFP will be expressed only if a donor-derived cell that has an active insulin promoter is fused to a recipient cell. Although male pancreatic β cells were found in the islets of recipient mice, no GFP expression was observed [71]. The investigators concluded that BMCs can differentiate toward a pancreatic endocrine β -cell phenotype *in vivo* without cell fusion.

BM-derived pancreatic islet cells were shown to be functional in a mouse model of chemically induced diabetes. Streptozotocin (STZ)-treated mice have destruction of their pancreatic β cells with subsequent lack of insulin and resultant hyperglycemia. When STZ-treated mice were transplanted with c-Kit⁺ BMCs, a marked reduction of serum glucose occurred within 4–7 days post-transplant [72]. Although the investigators saw donor-derived insulin-producing cells in the pancreas, it was thought that the decrease in hyperglycemia was not due directly to development of BM-derived β cells incorporating into the pancreas, but rather to stimulation of endogenous cells to proliferate and produce insulin. The reasons were (a) the amelioration of hypoglycemia resulted in 4–7 days post-transplant, at which time no insulin-positive, GFP⁺ cells were detectable yet in the islets; (b) BrdU costaining showed that the majority of proliferating cells in the pancreas post-BMT were GFP⁺

cells; and (c) there were not many insulin-producing GFP⁺ cells over time [72]. However, it may be that transgene (GFP) expression was turned off, and it would have been helpful to check for BM-derived cells using more reliable markers such as the Y chromosome.

BMCs to Kidney

Sex-mismatched transplantation models have been used to assess BM-derived renal tubular epithelial cells. Lethally irradiated female mice receiving BMT from male donors have kidney cells that costain for the Y chromosome and for the epithelial cell-binding lectins *Ricinus communis*, *Lens culinaris*, and *Pisum sativum* [73]. These cells also express the cytochrome P450 enzyme CYP1A2, suggesting functionality. In kidney biopsies from male patients who had been transplanted with kidneys from female donors, 1.8%–20% of renal tubule epithelial cells were BMC-derived [73]. This finding was corroborated by studies in which BM from a transgenic *lacZ*-positive mouse was transplanted into sublethally irradiated mice and ischemic renal injury was induced 16 weeks post-transplantation [74]. BM-derived tubules were identified by costaining for β -gal and megalin, a specific renal proximal tubule marker. The incorporation of BMC into renal tissue only occurred when Lin⁻, c-Kit⁺, and Sca-1⁺ hematopoietic progenitors were used as donor cells and not when more mature (Lin⁺) cells were injected into recipient mice [74].

BMCs to Central Nervous System (CNS)

The main classes of cells in the CNS are nerve cells (neurons) and glial cells. Glial cells are divided into two major classes: macroglia (astrocytes, oligodendrocytes) and microglia. Neural and macroglial cells derive from the ectodermal embryonic layer. Microglia, which represent approximately 5%–20% of glial cells, are believed to be derived from bone marrow [75, 76]. Historically, the mammalian CNS was considered to belong to a class of nonrenewable tissues [77], but this long-standing principle of neuroscience is being challenged by studies carried out in the last decade, which have shown neuronal progenitor cells that are capable of cell division in the CNS. Although the function and longevity of these cells are still being investigated, unambiguous evidence for adult neurogenesis in mammals has been demonstrated in the dentate gyrus and olfactory bulb [77, 78]. Recently, dividing neuronal cell populations have been found in the spinal cord as well [77]. Several papers (reviewed below) suggest that BMC can differentiate into CNS cells, including neurons, oligodendrocytes, and astrocytes, both *in vivo* and *in vitro*.

In vitro studies on BMCs to CNS plasticity focus on the capacity of the stromal BMC population to self-renew and to

differentiate into neuronal phenotypes. Rat stromal cells can adopt a neuronal-like phenotype after being cultured in medium containing butylated hydroxy anisole (BHA), dimethylsulfoxide (DMSO), and β -mercaptoethanol. The cells display long cell processes and express the markers neuronal-specific esterase and neurofilament medium (NFM). Human MSCs differentiate into cells that morphologically resembled progenitors of neuronal cells and expressed neural markers when grown in medium containing 3-isobutyl-1-methylxanthine (IBMX) and cAMP [79, 80]. Using culture medium containing growth factors (epidermal growth factor [EGF] or brain-derived neurotrophic factor [BDNF]) and retinoic acid, or coculturing with fetal mouse mesencephalic cells, Sca1⁻ BM cells express neuron (NeuN) and astrocytic (glial fibrillary acidic protein [GFAP]) markers [80]. Similar results are obtained with CD34⁻ BMCs cultured *in vitro* in the presence of fibroblast, epidermal, and nerve growth factors (FGF, EGF, NGF) and retinoic acid [81].

Under cytokine stimulation, MAPCs change morphologically, resembling astrocytes, oligodendrocytes, and neurons. In addition, when introduced into early blastocysts, MAPCs contribute to the formation of neurons and astrocytes throughout the brain, including the cortex, striatum, hippocampus, thalamus, and cerebellum. However, when these cells are infused intravenously into sublethally irradiated adult mice, no significant engraftment of mouse MAPC occurs in the brain [82].

Human fetal hematopoietic stem/progenitor CD34⁺/CD133⁺/CD3⁻ BMCs generate neuronal progenitor-like cells that express nestin and BMP-2 when they are cultured in astrocyte-conditioned medium or are cocultured with astrocytes. This BMC subpopulation, enriched for neuronal progenitor cells, could also differentiate into astrocytes (GFP⁺; S100⁺) under defined culture conditions [83]. Reviewing the *in vitro* studies, it must be pointed out that different laboratories use different culture conditions and start from different subpopulations of BMCs. This is not necessarily a negative aspect of these studies; rather, the data confirm that there are several pathways to trigger BMCs to differentiate *in vitro* into neuronal-type cells. The ability of BM-derived cells to differentiate into cells of the CNS has been assessed *in vivo* as well. BM-derived cells expressing the astrocyte marker GFAP develop in both the white and gray matter of the brain of myeloablated murine BMT recipients [76]. Donor-derived GFAP⁺ cells also form in the CNS after direct administration of MSCs into the lateral ventricles of 3-day-old mice. In these same recipients, donor-derived cells expressing neuron-specific neurofilament protein were present in the reticular formation of the brain stem [84]. Similarly, in a patient who had received a BMT as an infant, donor-

derived neuron-like cells (7 in 10,000) were found in the brain after 1 year [85]. These cells occurred in clusters, suggesting that a single progenitor cell may have expanded before differentiation.

Injection of the cytokines G-CSF and SCF after BMT may stimulate acquisition of BM-derived cells in the CNS of 1-day-old pups or adult mice [86, 87]. Cytokine-treated transplanted animals have a threefold increase in BM-derived neuron-like cells in the temporal cortical area and approximately a twofold increase in the olfactory bulb compared with untreated transplanted mice. Cytokine-treated mice that received transplantation at birth displayed a higher number of BM donor-derived neurons than mice that were transplanted in adulthood [88].

To test which BMCs can differentiate into neuronal cells, c-Kit⁺ BMCs were transplanted intracerebrally into neonatal mouse brains. After 1 week, BM-derived cells expressing the oligodendrocyte marker (O4) were present in the ependymal layer, and those with neuronal character (β -III tubulin) were in the subventricular zone, striatum, and cerebral cortex [89]. In contrast, c-kit⁻ BMCs did not differentiate into cells with a neuronal phenotype.

Studies marking BMCs with retroviruses have also identified BMC-derived cells in the CNS. Whole BMCs cultured for 48 hours in interleukin-3 (IL-3), interleukin-6 (IL-6), and SCF, and subsequently transduced with a retroviral vector encoding GFP, were injected into lethally irradiated mice. Within 1 month, rare GFP⁺ NeuN-expressing cells were detected in the olfactory bulb; at 12–15 months post-transplant, up to 0.1% fully developed cerebellar Purkinje neurons expressed GFP and the Purkinje cell antigen calbindin-D28K [90]. These cells displayed the typical morphology of Purkinje cells with extensive dendritic arborization and actively expressed γ -aminobutyric acid (GABA)-synthesizing enzyme, thereby strongly suggesting neurotransmitter activity [90].

A single Purkinje cell was also noted in a different experiment in which GFP⁺ BMC hematopoietic progenitor (KTLS) cells from a transgenic donor were transplanted into a lethally irradiated host. In this experiment, only one Purkinje cell was found in four mice analyzed [50]. Subsequent studies show that the nuclei of the BMCs were reprogrammed to express Purkinje-specific genes after cell fusion of a BM-derived cell with a mature Purkinje cell [91, 92]. Despite this unexpected mechanism of formation, these data suggest that BMCs can be used as vehicles for gene therapy to the brain.

BMC incorporation into neuronal tissues is enhanced after tissue injury. When MSCs are injected into the striatum and cortex of previously injured mouse brain near a site of cerebral artery occlusion, the cells enter into the injured area

and express neuronal and astrocytic protein markers [93]. Similarly, when MSCs are injected intravenously into rats that have been subjected to traumatic brain injury, they migrate into the injured area [94]. Of note, the rats that received BMCs showed reduced motor and neurological defects compared with rats that did not receive cells. Although donor-derived cells in the brain parenchyma expressed NeuN or GFAP, suggesting that they may function as neuronal and astrocytic cells, these cells did not re-establish the normal tissue cytoarchitecture [94]. Therefore, it is unlikely that they were directly responsible for the functional outcome observed. More likely, the BMCs facilitated tissue repair by indirect means.

Under pathological conditions, circulating BMCs can enter the brain and may actively participate in the renewal of CNS tissue. When middle cerebral artery occlusion was performed on mice that had been transplanted previously with GFP⁺ marrow cells, donor-derived cells appeared in the injured tissue within 3 days of the ischemic injury [95]. Donor-derived endothelial cells (assessed by colocalization of GFP and the endothelial markers Willebrand factor, CD31, and IB4 lectin) were present, thereby suggesting that BM may facilitate tissue repair by contributing to neovascularization. By days 7–14 post-injury, BM-derived cells expressing NeuN were found in the ischemic zone [95].

Human MSCs also differentiate into cells with markers of astrocytes, oligodendroglia, and neurons after direct injection into rats that have undergone cerebral infarction. As with administration of murine MSCs, mice receiving human MSCs show functional improvement even though the human MSCs did not establish new neuronal circuits with the host, again suggesting that these cells facilitate functional restoration indirectly [109].

BMCs have been tested for their ability to repair spinal cord damage in a rat model of demyelination, in which oligodendrocytes that maintain myelination are destroyed by local injection of ethidium bromide followed by X-irradiation. Administration of BMCs, either directly into the lesion or intravenously, was associated with remyelination of the neuronal axons, and the conduction velocity of the remyelinated axons was increased [96, 97]. However, the derivation of the new myelin-forming cells from the donor BMCs was only assessed by the cellular morphology of the donor cells in the areas of remyelination. It cannot be ruled out that the BMCs indirectly enhanced remyelination of the axon fiber by improving the recovery of endogenous myelin-forming cells. These results are consistent with those carried out by others [98, 99]. For example, injection of MSCs into injured spinal cords of paraplegic rats was associated with improved recovery of the paraplegic ani-

mals, even though the MSCs did not show significant differentiation into neuronal-like cells [100]. Taken together, these data suggest an important new concept: Even though MSCs are not able to replace damaged neuronal tissue in the spinal cord, they may be useful as accessory cells to facilitate healing, perhaps by producing trophic factors, cytokines, or other restorative factors.

Most of the *in vivo* studies demonstrating plasticity of BMCs into neuronal-lineage cells, in rodents as well as in humans, are based on the coexpression of donor-derived markers (Y chromosome or GFP) and one or more neuronal or glial proteins. However, there are several concerns regarding the specificity of the antibodies used, as well as technical problems related to microscopic detection of single cells expressing multiple fluorescent-labeled markers [78, 101]. In some cases, the neuronal protein expression pattern was supported by specific neuronal morphology *in vivo* [50, 90]. However, even if BMCs can be induced by their local microenvironment to express specific neuronal genes and assume a specific morphology, the functionality, such as the capacity to integrate into the neuronal transmission network, has not been shown. Data are consistent with BM-derived cells providing a “neuroprotective effect” on the existing cells, perhaps by activating endogenous healing mechanisms—including revascularization.

Even though definitive scientific evidence that BMCs can be used to treat CNS dysfunction is lacking, doctors have started to use BM-derived cells to treat patients. To date, data from a single phase I trial have been published. In this trial, patients with the severe neurodegenerative disease amyotrophic lateral sclerosis (ALS) were injected intrathecally with BMCs. After 6–12 months, none of the patients had side effects, yet significant clinical efficacy was not shown [102]. Related studies are under way in several clinical centers.

CURRENT CHALLENGES IN PLASTICITY STUDIES

Cell–Cell Fusion

Reports of *in vitro* and *in vivo* cell–cell fusion suggest that somatic cell fusion may be misleading scientists to conclude that there are stem cells in the bone marrow that are more pluripotent than previously believed [52, 53, 103, 104]. For example, when BMCs from a female mouse that was transgenic for both GFP and puromycin resistance genes were cocultured with a male embryonic stem cell line on gelatin-coated plates, puromycin-resistant clones developed that were morphologically similar to embryonic stem cells and could be induced to differentiate into cardiac myocytes and neuronal cells. This had initially suggested that the donor-derived BMCs had differentiated into embryonic stem cells. However, when DNA ploidy was analyzed, the cells were

tetraploid ($n = 11$) or hexaploid ($n = 2$), suggesting that they had resulted from spontaneous fusion between the BMCs and embryonic stem cells [103]. As described in the previous section on differentiation from BM to liver in FAH-deficient mice, wild-type BM-derived cells can take on the phenotype and function of hepatocytes by cell-cell fusion [103, 104]. Thus, it is possible that some or all of the *in vivo* differentiation that has been referred to as BMC plasticity may be due to fusion of differentiated BM-derived hematopoietic cells, perhaps macrophages, with mature tissue-specific cells. This issue was addressed *in vivo* using mice with normal liver function and the Cre/lox system. Stoplox- β -gal transgenic mice that contain *lacZ* downstream of stop codons flanked by loxP sites were lethally irradiated and injected intraperitoneally with BMCs from mice that ubiquitously express Cre recombinase and GFP [91]. If a cell from the donor and recipient fused, then the Cre enzyme would permanently remove the Lox P-flanked DNA, allowing expression of the *lacZ* gene. β -gal⁺ (fused), GFP⁺ cells were found in the brain, heart, and liver of BMT recipients 2 and 4 months post-transplantation. Importantly, no GFP⁺, β -gal⁻ cells were found in recipient tissue, and the hematopoietic compartment was largely reconstituted with donor cells. In a second experiment, donor mice expressed Cre recombinase from the CD45 locus, so that cells that express CD45, or once expressed CD45, also express Cre recombinase. These cells were used as donors for BMT into stoplox- β -gal reporter mice. Ten months post-transplant, all four BMT recipients had β -gal⁺ hepatocytes, half had β -gal⁺ cardiomyocytes, and one mouse had β -gal⁺ Purkinje cells [91]. Thus, BMC engraftment into these tissues is at least in part, if not entirely, due to fusion.

LACK OF REPRODUCIBILITY OF FINDINGS

Several reports in which BM-derived cells do not differentiate into epithelial or neural cells have called into question the validity of the initial findings. For example, Castro et al. [105] failed to detect marrow-derived, neuronal-like cells, or microglial cells in the brains of lethally irradiated mice that received bone marrow from ROSA26 mice with a *lacZ*-containing transgene. There are many possible explanations for the disparate findings. For example, because the experiments were not performed using identical mice and detection techniques as those in which BM to epithelial engraftment occurred, the data could be due to false negative findings, because ROSA26 transgenic mice show transgene instability in several tissues, and β -gal detection is sensitive to fixation and staining conditions.

Wagers et al. [50] failed to reproduce the results obtained in single-cell transplantation assay by Krause et al. [49]. Despite differences in the donor cell population used, they did show a very low frequency of liver cells ($1/6.7 \times 10^4$) and

Purkinje cells ($1/1.3 \times 10^7$) that were derived from a single KTLS BMC.

Need for Accurate and Sensitive Techniques

Technical problems still persist and can be misleading. Most of the *in vivo* and *in vitro* work establishing plasticity of BMCs is based on the coexpression of donor cell markers (e.g., Y chromosome FISH, GFP-expressing cells, and β -gal-expressing cells). When samples are analyzed using stringent conditions and proper controls, techniques such as immunohistochemistry, immunofluorescence, and FISH can be very revealing. Optimal methods for costaining individual cells are still being established, and false positive results can be a serious problem. When possible, all results should be verified using alternative approaches.

DISCUSSION

Publications on the potential plasticity of BMCs are significant and exciting, yet there are several questions that need to be answered before these cells can be considered to have therapeutic relevance. The biological mechanisms for BMC plasticity are at the center of an open debate, with multiple hypotheses being proposed for this plasticity:

- BMCs could be the source of specific stem cells present in different tissues. Data showing that BMCs give rise to oval cells, satellite cells, and type II pneumocytes support this hypothesis.
- Fusion could explain why BM-derived cells are able to take on diverse tissue phenotypes. Even though fusion alone is unlikely to underlie all of the plasticity data, it cannot be ruled out that, under selective pressure such as acute organ damage, BM-derived cells may fuse with other cell types.
- BMCs could be genetically reprogrammed when they are exposed to a different microenvironment. This phenomenon has been described as “transdifferentiation.” Transdifferentiation occurs in fish and amphibians, and nuclear reprogramming can occur in mammalian cells under certain experimental conditions [106]. However, no study to date has demonstrated that transdifferentiation underlies adult stem cell plasticity.
- Finally, a truly pluripotent stem cell may persist through adulthood. This hypothesis is supported by the findings of Jiang et al. [82], who demonstrate that MAPCs contribute to most cell types when injected into the blastocyst.

An essential area of future investigation is clonality studies, showing that a single, well-characterized marrow-

derived cell gives rise to mature, functional cells of multiple tissues. We need to understand at a single-cell level what part BMCs play in the cell turnover of various organs in normal and diseased states and the biological events that guide these phenomena. It is hoped that in the near future we will begin to characterize plastic BM-derived stem cells in the rigorous manner that hematopoietic stem cells have been characterized. At the same time, studies on epigenetics will help us determine what commitment means at a nuclear level.

Most of the collected data, showing that BMCs give rise to different tissue-specific cells, are based on immunohistochemistry and on visible morphological changes of the engrafted cells. This approach, as discussed above, could be insufficient for assessing plasticity. Even when BMCs are shown to express specific genes and modify their shape, the functionality and integration into the host tissue-network are not guaranteed. Nevertheless, there are some reports that unequivocally demonstrate that BMCs can engraft in non-hematopoietic tissues, integrate into the host tissue, and work together with endogenous cells. This is the case of the reprogrammed BM-derived hepatocytes described by Lagasse et al. [51], and the BMC-derived myocardial cells described by Orlic et al. [107]. In addition, the fact that a boy transplanted with allogeneic bone marrow displays an unexpectedly mild form of muscular dystrophy contributes to our belief that BMCs may help ameliorate pathological conditions in vivo

[110]. This important therapeutic effect need not be due to unexpected levels of bone marrow plasticity. For example, various studies show that administration of BMCs can stabilize CNS disease by stimulating the activity of the endogenous cells instead of by replacing the damaged tissue [98, 100].

It is important to note that, even though the basic science surrounding BMC plasticity is at an early stage and lacks conclusive scientific evidence, clinical trials using BMCs have started for some diseases that are particularly difficult to treat effectively. We now proceed with a balance of caution and excitement. On the one hand, when possible, adequate preclinical studies must be performed to assess safety and efficacy before human trials proceed. On the other hand, patients are of course anxious to know whether this will work in humans. Currently, several centers are conducting clinical trials to assess whether administration of marrow-derived cells to patients following myocardial infarction can lead to improved outcome. Despite promising clinical outcomes, as of this writing, no randomized controlled trials have been published.

In conclusion, scientists need to make an effort to understand the biological mechanism(s) of BMC plasticity and to characterize, or give an "identity" to, plastic BM subpopulation(s). At the same time, researchers need to ask themselves whether these phenomena, even if they do not occur physiologically, can be manipulated for therapeutic use.

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