Brief Definitive Report

Cells Capable of Bone Production Engraft from Whole Bone Marrow Transplants in Nonablated Mice

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Summary

Allogeneic and autologous marrow transplants are routinely used to correct a wide variety of diseases. In addition, autologous marrow transplants potentially provide opportune means of delivering genes in transfected, engrafting stem cells. However, relatively little is known about the mechanisms of engraftment in transplant recipients, especially in the nonablated setting and with regard to cells not of hemopoietic origin. In particular, this includes stromal cells and progenitors of the osteoblastic lineage. We have demonstrated for the first time that a whole bone marrow transplant contains cells that engraft and become competent osteoblasts capable of producing bone matrix. This was done at the individual cell level in situ, with significant numbers of donor cells being detected by fluorescence in situ hybridization in whole femoral sections. Engrafted cells were functionally active as osteoblasts producing bone before being encapsulated within the bone lacunae and terminally differentiating into osteocytes. Transplanted cells were also detected as flattened bone lining cells on the periosteal bone surface.

Key words: osteoprogenitors • osteoblasts • bone • bone marrow transplants • nonablated

Marrow transplants are routinely used to correct a wide variety of diseases. In addition, autologous marrow transplants potentially provide opportune means of delivering genes in transfected, engrafting stem cells. However, relatively little is known about the mechanisms of engraftment in transplant recipients, especially in the nonablated setting and with regard to cells not of hemopoietic origin. In particular, this includes stromal cells and progenitors of the osteoblastic lineage.

The bone marrow is encompassed by bone, a rigid structuring matrix that is subject to perpetual remodelling through the combined actions of osteoclasts, which erode matrix, and osteoblasts, which secrete it. Although osteoclasts are of hemopoietic origin (1, 2), osteoblasts are believed to be the progeny of mesenchymal stem cells (3, 4). Differentiation involves the formation of fibroblast-like progenitor cells, which can also give rise to cartilage, muscle, fat cells, tendon fibroblasts, and possibly other fibroblast types (5–7). Cells committed to the osteoblast lineage can be found in the periosteum as well as in the endosteum and the bone marrow (7–11).

Osteoblasts are organized in layers at the bone surface, working in a coordinated fashion to secrete new bone matrix. This matrix subsequently mineralizes extracellularly. When osteoblasts become trapped in newly formed mineralized matrix and remain viable, they further differentiate into osteocytes. Osteocytes, although they have no opportunity to divide or secrete further appreciable quantities of bone matrix, play a significant role in the regulation of bone matrix turnover. These cells lie in small cavities or lacunae within the bone matrix, and are connected to other osteocytes through gap junctions at the end of cell processes, which radiate through tiny channels, or canaliculi. Other osteoblasts become flattened on the bone surface, and are known as bone lining cells.

In this study, we have used a male into nonablated female transplant model to show that at the individual cell level in situ a whole bone marrow transplant contains cells that engraft, differentiate, are capable of bone formation, and reside within the bone tissue. Significant numbers of donor cells were detected by fluorescence in situ hybridization (FISH) in whole femoral sections, as both osteocytes encapsulated by mineralized matrix and residing within the bone lacunae and as flattened bone-lining cells in the periosteum.

Materials and Methods

Mice. 6–8-wk-old BALB/c H-2D mice were purchased from Charles River Labs. and were housed in a conventionally clean facility for at least 1 wk. All mice received mouse chow and acidified water ad libitum. The BALB/c H-2D strain was chosen because

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of its negligible immunoreactivity to the H-Y (Y chromosome–associated histocompatibility) antigen (12, 13).

Cell Suspensions and Transplants. Marrow was collected by flushing femurs and tibiae with cold PBS supplemented with 5% heat-inactivated FCS (Hyclone Labs.). The cells were filtered through a 40-μm filter (Becton Dickinson), washed, and resuspended for injection in 0.4 ml of PBS per recipient.

Transplants involved a single or three daily intravenous infusions into nonablated female recipients of the same age. After 6 wk or 6 mo, the presence of donor cells in the marrow was quantitated by Southern blot analysis and the spatial distribution of engrafted cells in the bone tissue was analyzed on whole femoral marrow sections using FISH.

Southern Blot Analysis. Marrow DNA extracts were prepared, digested, and separated by gel electrophoresis, and analyzed by Southern blot as previously described (14). The presence of Y chromosome–specific DNA sequences was assessed using a pY2-cDNA probe (donated by I. Lemischka, Princeton University, Princeton, NJ) (15). Sample loading variability was assessed and adjusted by reprobing membranes with IL-3 cDNA (donated by J. Ihle, DNAX, Palo Alto, CA). Probes were labeled with ^{32}P. The percentage of male DNA was quantified by PhosphorImager (Molecular Dynamics) analysis.

FISH. In situ detection of individual cells using FISH on sections of paraffin-embedded whole murine femurs was done as previously described (16). In brief, mice were perfused with 4% paraformaldehyde at physiological pressure into the descending aorta. 5-μm sections were permeabilized using proteinase K enzymatic digestion. Sections were hybridized with a digoxigenin-labeled Y chromosome–specific painting probe (17) and stained using antidigoxigenin rhodamine-Fab fragments. Levels of autofluorescence were determined by counter-staining in 0.2 μmol DAPI (4,6-diamidino-2-phenylindole) (see Fig. 1 d). Sections were mounted in Vectashield (Vector Labs.).

Results

To accurately assess the extent of donor chimerism and the histological location of engrafted cells within the bone tissue, cells of donor origin were detected by FISH, using a Y chromosome–specific painting probe, in whole femoral sections.

Distribution of Engrafted Cells. 6 wk after a marrow transplant, cells of donor origin were detected as osteocytes encapsulated in lacunae (Figs. 1 and 3 A). As osteocytes are terminally differentiated bone cells within the bone matrix itself, these cells could not have been transplanted during the original infusion but must be the progeny of engrafted, functionally active donor cells. In addition, donor cells were evident both as flattened lining cells on the periosteal bone surface and as cells within the endosteal growth region (Fig. 3, D and G). It is also important to note that because whole marrow was transplanted, a significant proportion of marrow cells in the recipients were also of donor origin (refer to the legends of Figs. 1 and 3 for exact proportions).

A technical limitation of FISH is the removal of the actual bone matrix during the denaturation process. As a consequence, cells at the endosteal marrow surface were not

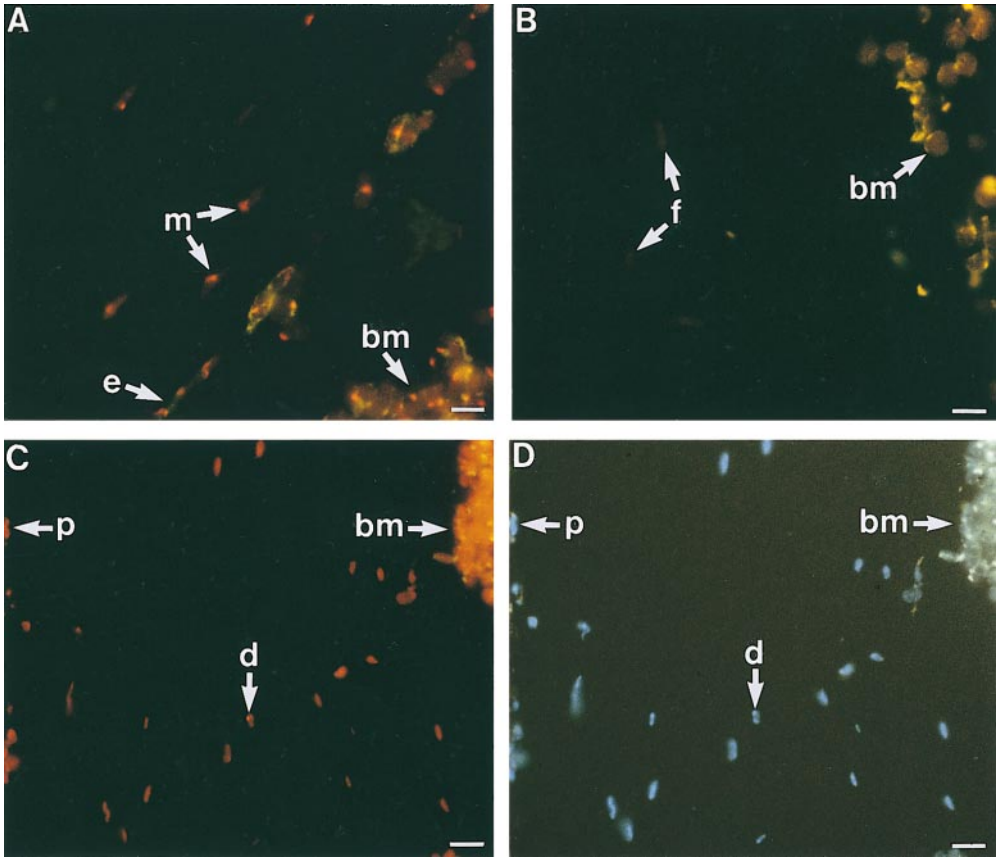

Figure 1. FISH of femoral marrow sections. Panel A, male control showing positive Y chromosomes (m; deep red/orange). Panel B, female control (f); autofluorescence appears yellow. Panel C, transplant recipient 6 wk after transplant of 1.2×10^8 male marrow cells. Sections are viewed using a dual red and green filter, and therefore have no counter-staining. Marrow chimerism was $23.9 \pm 2.7\%$ ($n = 5$), as determined by Southern blot analysis (Fig. 2). Panel D, same as C viewed using a DAPI filter. Note complete lack of auto fluorescence in positive cell. bm, bone marrow; d, positive bone cell of donor origin; e, endosteum; p, periosteum. Bars, 20 μm.

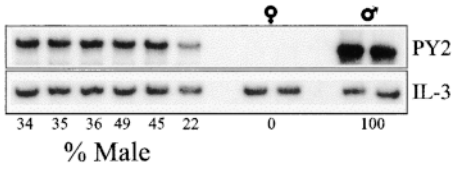

Figure 2. Percentage of male DNA in marrow of individual recipient female mice determined by Southern blot. Male was taken as 100% and female as 0%. Loading variability was corrected using an IL-3 probe.

scored, avoiding any possible confusion between positive bone marrow cells and positive bone cells once the bone matrix was removed. To confirm that the cells analyzed were both of donor and bone origin, serial, longitudinal femoral sections were cut and every second section was stained by FISH and every alternate section was stained with hematoxylin and eosin (H and E). Individual cells could then be identified in the bone lacunae, periosteum, or endosteal growth region on the H and E sections (Fig. 3, C, F, and I). Due to the thickness of each section (5 μm), a significant number of cells were present in both the FISH and serial H and E sections. For ease of identification, these cells are pictorially represented in each figure (Fig. 3, B, E, and H).

Engraftment Period. Bone cells of donor origin were easily detected both 6 wk and 6 mo after transplant, and no difference in the incidence of these cells was observed at either of these time points. In all sections analyzed after a transplant of 120–180 $\times$ 10^6 marrow cells (resulting in 19.2 $\pm$ 2.9 to 31.3 $\pm$ 2.5% marrow chimerism), at least one positive cell was detected. In greater than two-thirds of the sections analyzed (n = between 2 and 9 sections for 3 and 2 individual mice at 6 wk and 6 mo, respectively), three or

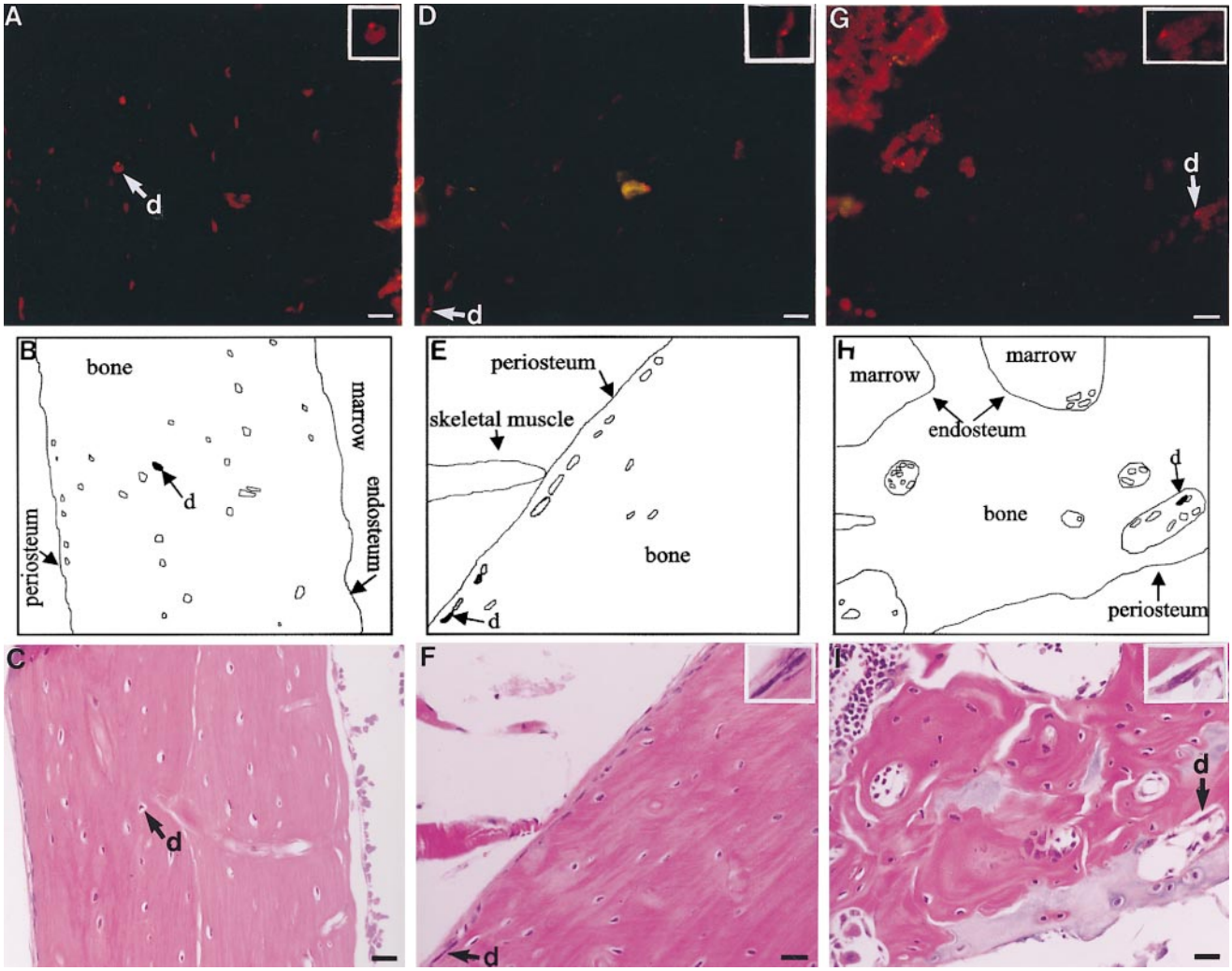

Figure 3. Femoral marrow sections from female recipients 6 wk after transplant of 1.8 $\times$ 10^8 male marrow cells. Marrow chimerism was 31.3 $\pm$ 2.5% (n = 13), as determined by Southern blot analysis. (A, D, and G) FISH labeling for the Y chromosome-specific probe; d, positive bone cells of donor origin (enlarged in the inserts). A, osteocyte; D, bone-lining cell; G, bone cell. (B, E, and H) Map of cells present both in A, D, and G and the respective H and E–stained serial sections. C, osteocyte of donor origin within a bone matrix lacuna. F, bone-lining cell of donor origin (enlarged in the insert) within the periosteum; I, bone cell of donor origin (enlarged in the insert) within the endosteum growth region. Bars, 20 μm.

more positive cells were detected. The maximum number of positive cells observed in the bone matrix and the periosteal region of any individual, centrally cut longitudinal section was 8, of which a significant number were in the matrix itself. With a section being 5 μm thick, and the central region of a femur from a 12-wk-old mouse ~2-mm-thick, there is a conservative estimate of up to 3,200 of these positive cells per femur. A section contained 2,870 ± 114 (n = 8) osteocytes, and therefore there was a maximum of 3 donor cells per 1,000 osteocytes.

Even up to the age of 6 mo, a mouse grows significantly. Between the ages of 6 and 12 wk, the period of the 6-wk transplant, this growth resulted in a significant 16.5% increase in body weight (18.3 ± 0.2 g and 21.9 ± 0.4 g, SEM, respectively; $P < 0.001$ using a Wilcoxon rank-sum test, n = 15 and 13 respectively). During this period of time, bone growth, or specifically bone remodelling by resorption and formation, resulted in a 30.3% increase in femoral mass (30.4 and 43.6 mg, respectively), as well as a significant increase in size (Fig. 4). Consequentially, by 12 wk of age there is essentially complete turnover of the bone present at 6 wk of age, allowing osteocytes of donor origin to be found anywhere within the bone matrix. In addition, mice transplanted at 6 wk of age and analyzed at 6 mo of age would have many complete turnovers of bone matrix during the transplant period. This suggests that a cell of donor origin detected as an osteocyte in the bone matrix 6 wk after transplant is extremely unlikely to be present 6 mo after transplant. Cells present in the bone matrix 6 mo after transplant are most likely to have been derived from cells that were not actively producing bone 6 wk after transplant; instead, the donor osteocytes detected 6 mo after transplant probably arose from an originally transplanted osteoprogenitor.

Discussion

Our data demonstrate that whole marrow contains cells of the bone lineage that can engraft post-intravenous infusion, form bone, and give rise to osteocytes and bone lining cells. These are easily detectable in the femur 6 wk and 6 mo after transplant into nonablated mice. This occurs long-term in vivo, without any in vitro culturing manipulation. The long-term stability in the incidence of bone cells of donor origin is consistent with the stable bone marrow chimerism that we have previously observed over this period of time (18).

Figure 4. Femurs isolated from a 6-wk-old (top) and a 12-wk-old (bottom) mouse, demonstrating a period of substantial growth. Bar, 2 mm.

The data support the suggestion by Pereira et al. (19) and Prockop (20) that transplanted cells with bone forming capabilities engraft in the marrow of recipient mice and participate in normal biological turnover, in which the marrow provides a continual source of osteoblastic progenitor cells. The data also supports the hypothesis proposed by Horwitz et al. (21) that whole marrow contains enough of these cells to be able to replace sufficient osteoblasts to be clinically useful in diseases such as osteogenesis imperfecta, where marrow ablation is standard preparative treatment. Other strategies for the therapeutic use of putative "mesenchymal stem cells" have involved the direct injection of isolated and culture-expanded bone marrow cells into bone lesions or joints. These techniques were shown to promote lesion repair and joint resurfacing at the site of injection but did not have systemic effects (7, 22).

Marrow stromal cells produce bone matrix when placed in diffusion chambers and implanted into the peritoneum (9, 10). Other studies have shown that fibroblast colonies, or clones originating from single stromal cells (CFU-f), have high osteogenic potencies (21) and can form bone (23). However, stromal cells are not repopulated in radiochimeras reconstituted by marrow transplantation (24–28). If marrow fibroblasts are to be used for clinical therapies, transplanting them so that they engraft and are active may be a major problem (29). Piersma et al. (30) have subsequently demonstrated that CFU-f could be repopulated in an irradiated host when infused intravenously as part of a whole marrow transplant, although the exact population being assayed remains controversial. Keating et al. (31) showed that the hemopoietic stromal microenvironment, defined as the adherent layer of human long-term marrow cultures grown from patients who received normal marrow transplants, progressively became donor with time after transplant. However, Simmons et al. (32) and Lennon and Micklem (33) more recently showed that the stromal fibroblastic cells in marrow cultures made from human allogeneic transplant recipients and long-term murine chimeras were mainly or entirely host derived. All these studies were done in irradiated recipients, involved in vitro manipulation in order to discriminate donor versus host, and did not go on to show functional feed-out of transplanted donor cells in vivo. Therefore, it remains to be determined whether stroma, which has been considered non-transplantable via intravenous infusion, lodges within the marrow when part of a whole bone marrow transplant.

Recently, Pereira et al. (19) showed that 1–5 mo after a transplant into irradiated hosts of "mesenchymal cells" enriched by adherence to plastic, expanded in culture, and marked with the human COL1A1 minigene, there was widespread tissue distribution of connective tissue cells

containing and expressing the gene. Along with many other tissues, such as brain, spleen, marrow, and lung, the study showed positive signal for and expression of the gene by PCR and reverse transcriptase PCR from ground bone. However, these positive results could be due to many different cell types, and they do not definitively prove the presence of donor bone cells. In the studies of Pereira et al. (19), the irradiation of recipient mice was necessary to ensure engraftment, and hence limits clinical application of this strategy. Our data demonstrate that the need for isolation and purification of osteoblastic cells can be circumvented, and preparative marrow ablation treatments are not required. In addition, the data show conclusively that not only do these cells successfully engraft when included as part of a normal marrow transplant, but they are then functionally active, as only osteoblasts, which are actively producing bone, can become encapsulated within it and differentiate into osteocytes.

Previous studies have shown that marrow chimerism can be achieved in mice without the need for marrow ablation (14, 18). This study provides the first clear demonstration of the in vivo derivation of osteocytes from a whole marrow transplant into nonablated recipients. With the development of new methodologies and transgenic mouse models, future studies will reveal further critical information at the individual cell level, as well as possibilities in the treatment of bone disorders by bone marrow transplantation.

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