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Ablation of Proliferating Marrow with 5-Fluorouracil Allows Partial Purification of Mesenchymal Stem Cells

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ABSTRACT

The ability to identify and maintain mesenchymal stem cells in vitro is a prerequisite for the ex vivo expansion of cells capable of effecting mesenchymal tissue regeneration. The aim of this investigation was to develop an assay to enrich and ultimately purify mesenchymal stem cells. To enrich the population of mesenchymal stem cell-like cells, rats or mice were administered 5-fluorouracil (5-FU) in vivo. Limiting dilution analysis demonstrated that 5-FU-treated bone marrow had the potential to form colony-forming units-fibroblastic (CFU-F) at a 10-fold or sixfold enrichment compared to normal bone marrow in rats or mice, respectively. In vivo and in vitro differentiation assays supported the enrichment and purification effects. In vitro, bone marrow cultures from 5-FU-treated bone marrow demonstrated lineage-specific gene expression in lineage-specific medium conditions in contrast to the multilineage gene expression of control bone marrow cultures. In vivo implantation of 5-FU-treated cells that were not expanded in culture generated ossicles containing an intact bone cortex and mature hematopoietic components, whereas non-5-FU-treated bone marrow only formed fibrous tissues. Our results demonstrate that enrichment of a quiescent cell population in the bone marrow by in vivo treatment of 5-FU spares those undifferentiated mesenchymal stem cells and influences the differentiation of bone marrow stromal cells in vitro and in vivo. This prospective identification of a population of mesenchymal cells from the marrow that maintain their multilineage potential should lead to more focused studies on the characterization of a true mesenchymal stem cell. STEM CELLS *2006;24: 1573–1582*

INTRODUCTION

Identification of stem cells or early lineage cells is often dependent on cell surface markers that designate the expression of antigens associated with states of activation, function, or differentiation [1–3]. Because morphological identification of early lineage cells is not reliable, a detailed knowledge of molecular function, including enzymatic and secondary markers of differentiation, is necessary. Stem cell identification by definition relies on the demonstration of the production of lineage-committed progeny. For hematopoietic stem cells (HSCs), the expression of CD34, c-kit, and a complex combination of lineage-committed markers of mature lineages (lin^-) have served as the basis of stem cell isolation [4, 5]. However, to date, the most effective means to distinguish HSCs from progenitor cells is the use of relatively simple combinations of signaling lymphocyte activation molecule family receptors expressed on HSCs [6].

The use of in vitro assays for identification of stem cells has been challenging [7–11]. Markers change with time in culture and may be linked to the supportive niche that stem cells occupy. Experimental in vitro end points including long-term culture-initiating cells, very-long-term culture-initiating cells, and cobblestone area have been proposed as surrogate markers for in vivo function [12, 13]. In contrast, in vivo assays for function of HSCs are the most rigorous and reliable, the preeminent of which are defined using a competitive repopulation assay, also referred to as severe combined immunodeficient (SCID)-repopulating assays [14, 15].

Recently, the concept of mesenchymal stem cell transplantation for the treatment of a variety of developmental and acquired anomalies has gained significant attention [8, 16–19]. The ability to move forward with these therapies, however, is impeded by the inability to definitively identify and characterize the putative mesenchymal stem cell (MSC). Despite the lack of cellular and molecular tools to make these determinations, it is known that a cell population with MSC-like characteristics can be isolated based on its ability to adhere to plastic and may be partially purified by separation techniques [7, 20–22]. Although bone marrow stromal cells (BMSCs) have the potential to

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differentiate into specialized cells of mesenchymal origin, including adipocytes, chondrocytes, myoblasts, and osteoblasts [23–29], studies have demonstrated that normal BMSCs are quite rare. They are present at low frequency in bone marrow and may be found in the circulation in small numbers [30]. BMSCs can be expanded to great numbers by in vitro culture; however, such a step is time-consuming and expensive and risks cell contamination. Most important, as these cells gain or lose their differentiation potential during long-term culture, the ability to maintain or expand stem cells without adequate in vitro or in vivo assays hinders the ability to identify putative stem cells as true stem cells. For identification of HSCs, the answer has been to eliminate the tissue through lethal levels of radiation. For mesenchymal stem cells or their immediate progeny, this approach is not feasible because of the lack of an assay comparable to the competitive repopulation assay.

The ability to identify and maintain early mesenchymal cells with stem cell-like activity in vitro is a prerequisite for the ex vivo expansion of cells capable of effecting mesenchymal tissue repair. The aim of this investigation was to develop an in vivo assay for MSCs similar to the SCID-repopulating assay for HSCs. It has been previously demonstrated that in vivo treatment with 5-fluorouracil (5-FU), a nucleotide analogue that is incorporated into DNA during the S-phase of the cell cycle leads to cell death of cycling cells and enhances the osteogenic potential of stromal cells in vitro [10, 31]. We therefore sought to determine whether such a population of cells would maintain its stem cell-like activity in vivo prior to any cell culture and begin characterizing this enriched stem cell population.

Using in vitro limiting dilution analysis and in vivo implantation of cells derived from 5 FU-treated animals, we greatly enriched the cell population capable of forming bone in vivo and found that transplants of noncultured cells generated bone marrow-containing ossicles exhibiting cortical bone and mature bone marrow structures including adipocytes and chondrocytes. Our findings also provide evidence that 5-FU spared those undifferentiated mesenchymal stem cells in vivo and influenced the differentiation of BMSCs in vitro.

Materials and Methods

Animals

Marrow donor animals (8-week-old BALB/c mice and 7- to 8-week-old Fischer rats) were obtained from Charles River Laboratories (Wilmington, MA, http://www.criver.com). Animals were caged under standard conditions and fed a laboratory diet and tap water ad libitum. Care and use of the laboratory animals followed the guidelines established by the University of Michigan Committee for the Use and Care of Animals.

5-FU Treatment

One hundred milliliters (wt/vol) 5-FU (American Pharmaceutical Partners, Inc., Schaumburg, IL, http://www.appdrugs.com) was administrated intravenously via the lateral tail vein at a dose of 150 mg/kg (mice) or 50 mg/kg (rats) body weight 5 days prior to marrow harvest. Control animals were injected with an equal volume of 0.9% sodium chloride vehicle solution. Marrow donor animals were euthanized by CO_2 inhalation, followed by cervical dislocation, and the bone marrow was harvested under sterile conditions.

Bone Marrow Cell Preparation

The femora, tibia, and humera of donor animals were excised, and adherent tissue was dissected. The marrow was expelled using a flushing stream of Hank's buffer salt solution (Gibco, Grand Island, NY, http://www.invitrogen.com) delivered from a 5-ml syringe fitted with a 23-gauge needle. A single-cell suspension was obtained by gentle agitation through the syringe. Debris and remaining cellular aggregates were removed by passing the cell suspension over a 70-mesh nylon cell strainer (BD Biosciences, San Diego, http://www.bdbiosciences.com).

Cell Culture

Bone marrow cells were resuspended in alpha modified Eagle's medium (α-MEM; Gibco) supplemented with 15% fetal calf serum (FCS; Gibco), 1% penicillin and streptomycin, L-ascorbic acid-2-phosphate (50 mg/l; Wako Inc., Osaka, Japan, http://www.wako-chem.co/jp/english), and β-glycerophosphate (10 mM; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and plated into flat-bottom 96-well plates at varying cell numbers. On day 5, 50% of the medium was replaced with fresh medium, and the total culture medium was replaced every 3 days thereafter.

Limiting Dilution Analysis

Bone marrow cell suspensions were density centrifuged on 70% Ficoll. Log serial dilutions of the experimental marrow populations were placed into 10 to 20 replicate wells in a total of 200 μl of medium. Half the medium was replaced at weekly intervals. At the conclusion, the wells were stained with crystal violet. By enumerating each colony assay as "positive" ($\geq$7 cells clustered in one colony) or "negative" ($<$7 cells present in one colony) for colony-forming unit-fibroblast (CFU-F), the frequency of progenitor cells in the undiluted starting population was calculated (L-Calc Software; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). The number of cells required to form one CFU-F, which reflects the proportion of mesenchymal progenitor/stem cells in the entire bone marrow population, was then determined from the point at which the line crossed the 37% level. Based on a Possion's distribution of progenitor cells, $F_0 = 37\%$ corresponds to the dilution at which statistically there is one progenitor cell per well [31].

In Vivo Assessment of Multilineage Stem Cell Activity

BMSCs (5-FU-treated or control) were assessed for their potential to form bony ossicles after transplantation in immunodeficient mice. Bone marrow from 5-FU-treated and non-5-FU-treated rats or mice was collected individually. Numbers of nucleated bone marrow cells ranging from 0, 0.125, 0.25, 0.5, 1, 2, to 4 million were incorporated into a gelatin sponge (Gelfoam; Pharmacia & Upjohn, Kalamazoo, MI, http://www.pharmacia.com). These cell/scaffold constructs were transplanted subcutaneously into 5-week-old male mice (NIH-bg-nu-xid BR; Harlan Sprague Dawley, Indianapolis, http://www.harlan.com). The mice were anesthetized by intraperitoneal injection of an anesthetic cocktail (75 mg/kg Ketamine, 10 mg/kg Xylazine of body weight). A skin incision was made on the dorsal surface of each mouse, and four

subcutaneous pockets per mouse were created by blunt dissection. A single implant was placed into each pocket, and incisions were closed with surgical staples [17, 18].

Sample Harvest and Evaluation

The implants were harvested 5 weeks after surgery and fixed in aqueous buffered zinc formalin for 24 hours at 4°C. For microcomputed tomography (μCT) analysis, specimens were scanned at a 8.93-μm voxel resolution on an EVS Corporation μCT scanner (London, ON, Canada, http://www.biodevicesbiz.com), with a total of 667 slices per scan. GEMS MicroView software was used to make a three-dimensional reconstruction from the set of scans. A fixed threshold (1,500) was used to extract the mineralized bone phase and actual bone volume, and bone mineral density (BMD) was calculated. For histology, the specimens were decalcified for 3 days in 10% formic acid and embedded in paraffin, and 5-μm serial sections were prepared and stained with hematoxylin/eosin.

Proliferation and Differentiation Assays of Bone Marrow Cells

Cell proliferation was measured by a colorimetric method using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfonphenyl)-2*H*-tetrazolium (MTS tetrazolium) compound (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, http://www.promega.com). Bone marrow cell suspensions were density centrifuged on 70% Ficoll to remove red blood cells, and approximately 400,000 nucleated cells/well were seeded onto 96-well tissue culture plates. Cells were grown for various time points in α-MEM supplemented with 10% FBS and 1% Pen/Strep with the culture medium changed every other day. On the day of the proliferation assay, the culture medium was aspirated and replaced with 100 μl of fresh medium. Twenty μl of MTS reagent was added, and cells were allowed to incubate for 2 hours at 37°C in a humidified CO_2 (5%) atmosphere. The amount of soluble formazan product produced by the reduction of MTS by metabolically active cells was measured by a 96-well spectrophotometer at 490 nm absorbance.

For the in vitro osteogenesis assay, the cultures were incubated in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone (Decadron; Merck & Co., Inc., Whitehouse Station, NJ, http://www.merck.com), and 0.5 μM ascorbate-2-phosphate. The medium was changed two times per week for 3 weeks. The cells were fixed with 10% formalin for 20 minutes at room temperature and stained with 1% silver nitrate for 20 minutes at room temperature (von Kossa's staining) to identify mineralized nodules.

For adipogenesis, the cultures were incubated in DMEM that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 12 mM L-glutamine, 5 μg/ml insulin (Gibco), 50 μM indomethacin (Sigma), 10^{-6} M dexamethasone, and 0.5 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma). Fixed cells were stained with Oil Red (Sigma) in ethanol for 20 minutes at RT.

For chondrocyte differentiation, the cultures were incubated in DMEM that was supplemented with 15% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml TGF-β1 (BD Biosciences), 5 μg/ml insulin, 1×10^{-7} M dexamethasone, 0.4 mM proline (Gibco), 0.5 μM ascorbate-2-phosphate, 0.1 mM MEM-nonessential AA (Gibco). The medium was changed two times per week for 3 weeks. Fixed cells were stained with Safranin O (Sigma) in ethanol for 20 minutes at RT.

Table 1. Limiting dilution analysis and CFU-F formation demonstrate significant enrichment of mesenchymal stem cells in 5-FU-treated bone marrow

Species	5-FU treatment	Frequency of CFU-F
Rat	−	1/97,640 (1/128,766–1/74,029)
Rat	+	1/9,347 (1/9,895–1/8,831)[a]
Mouse	−	1/59,653 (1/103,276–1/34,451)
Mouse	+	1/10,106 (1/10,290–1/9,927)[a]

[a] $p < .05$.

Abbreviations: 5-FU, 5-fluorouracil; CFU-F, colony forming unit-fibroblast.

RNA Extraction, cDNA Synthesis, and RT-PCR Analysis of Various Differentiation Gene Markers in Bone Marrow Cells

To investigate gene expression in bone marrow cells after in vivo 5-FU treatment, various gene markers of differentiation were analyzed. RT-polymerase chain reaction (PCR) analysis using total RNA was performed in uncultured bone marrow cells and bone marrow cells cultured in specific differentiation medium at harvest and day 21. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and treated with 1 U of DNase I at room temperature for 15 minutes to digest any contaminating DNA. Aliquots containing 1 μg total RNA were used to prepare cDNA. The PCR was carried out with Taq DNA polymerase (Invitrogen). The reaction profile was 94°C for 30 seconds, 55–58°C for 1 minute, and 72°C for 1 minute. We used 35 cycles for all the assays. D-Glyceraldehyde-2-phosphate dehydrogenase was used as an internal control. PCR products were separated by electrophoresis in 1.8% agarose gels and visualized by ethidium bromide staining.

Statistics

The mean and standard derivation is reported at each group for bone volumes and BMD by μCT. The Student's t test was used to determine significant differences between groups. We applied curve simulations to calculate the frequency of CFU-F with the SPSS program (version 10; SPSS Inc., Chicago, http://www.spss.com). Differences were considered significant at $p < .05$.

RESULTS

Frequency of CFU-F

The aim of this study was to develop an in vivo correlate to the SCID-repopulating assay for MSCs (Fig. 1). Based on what is known in other stem cell systems, we reasoned that most MSCs at any given time would be cycling slowly and be of low density. Therefore, we developed a strategy to enrich for a population that contained cells with MSC-like activity and tested their potential in vitro and in vivo.

Initial investigations were performed to identify the frequency of CFU-F derived from rat and mouse bone marrow using standard limiting dilution methods. Based on Poisson

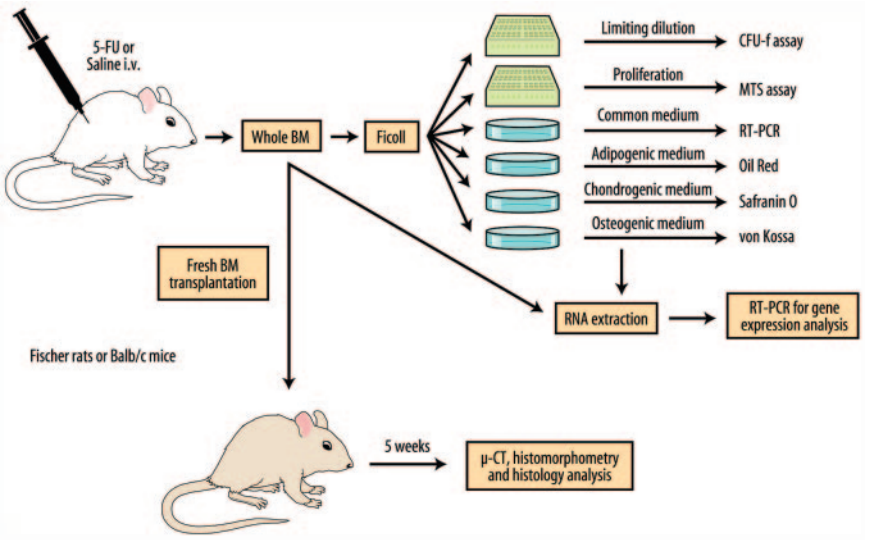

Figure 1. Schematic of the in vivo 5-FU treatment strategy to enrich cells for MSC characteristics in bone marrow. Fischer rats and BALB/c mice were injected intravenously with 5-FU. Bone marrow was harvested 5 days post-treatment and was either immediately transplanted to severe combined immunodeficient mice at 0.125, 0.25, 0.5, 1, 2, or 4 $\times$ 10^6 nucleated cells per implant or cultured ex vivo. The newly formed tissues were measured by μCT, and histologic analysis was performed to identify the constitution of the implants. In vitro evaluation included limiting dilution assay for CFU-F frequency, MTS for proliferation, and differentiation staining under specific induction medium. For gene expression studies, RNA from both fresh medium and BMSCs cultured in lineage specific differentiation medium were extracted and subjected to RT-PCR analysis. Abbreviations: 5-FU, 5-fluorouracil; BM, bone marrow; CFU-F, colony forming units-fibroblastic; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfonphenyl)-2*H*-tetrazolium; RT-PCR, reverse transcription-polymerase chain reaction.

distribution [32], the intersection of the corrected line with a 37% level demonstrated that under standard culture conditions, one of every 9.7 $\times$ 10^4 nucleated rat cells from control bone marrow had the capacity to form a CFU-F. In contrast, the frequency of CFU-F in mice was slightly higher, demonstrating a rate of one per 6.0 $\times$ 10^4 nucleated cells (Table 1). Treating animals with 5-FU led to a significant increase in the frequency of CFU-Fs. CFU-Fs generated from bone marrow cells increased to one of 9.3 $\times$ 10^3 nucleated cells in rats or one of 10.1 $\times$ 10^3 cells in mice. These data demonstrate that treatment of animals with 5-FU increased the frequency of CFU-F formation from bone marrow cells sixfold in mice and 10-fold in rats (Table 1).

In Vivo Determination of Multilineage Stem Cell Activity

Our findings were similar to a previous report and demonstrated that in vivo treatment of animals with 5-FU and subsequent separation in Ficoll increased the frequency of CFU-F in vitro [31]. At least two possibilities exist to account for these observations. The first is that the quiescent state of the cells spares them from the metabolic poison, or that following 5-FU treatment the stem cells have the capacity to expand in vivo. In either case, CFU-F frequency would increase.

To first verify that CFU-F numbers reflect stem cell activities, nucleated low-density donor marrow cells were isolated from rats or mice following in vivo 5-FU treatment. Recipient immunodeficient mice were immediately transplanted with limiting dilutions of low-density mononuclear cells in gelatin sponges over a dose range of 0–4 $\times$ 10^6 cells (Fig. 2A, 2B). After 5 weeks in vivo the tissues were recovered, radiographed, and prepared for histology. Gross inspection of 5-week in vivo samples demonstrated that transplants containing 5 $\times$ 10^5 cells formed small, avascular nodules (data not shown). For implants derived from 5-FU treated animals, the 2$\times$ 10^6 and 4$\times$ 10^6 cell groups formed organized tissue with visible vascular invasion. Sponge implants containing vehicle-treated marrow formed only whitish soft tissue nodules.

Histological examination of the control sponges transplanted without bone marrow cells resulted in only low levels of neutrophils and connective tissue ingrowth (data not shown). Implantation of 5 $\times$ 10^5 cells in either group also failed to demonstrate specific tissue regeneration and consisted of loosely organized connective tissue and residual collagen scaffold material (Fig. 2). Implantation of 1.0 $\times$ 10^6 cells from the 5-FU-treated rats produced small focal areas of hypertrophic cells that resembled cartilage-like tissue; however, molecular analyses did not support this morphologic finding (Fig. 2A). At 2 $\times$ 10^6 cells/implant the 5-FU-treated groups demonstrated significant new bone formation, adipocytes, and the recruitment of marrow. Further enhancement of bone/marrow organ development, including an intact cortex-like structures, medullary adipose tissue, and a mature hematopoietic marrow cavity was observed following implantation of 4 $\times$ 10^6 5-FU-treated cells. The extent of osteogenesis was dependent on the number of cells seeded, with the 4 $\times$ 10^6 nucleated cell groups demonstrating more intact cortex-like structure, thicker of trabeculae, and more bone marrow than those of 2 $\times$ 10^6 nucleated cell groups. Such bone formation was not observed when noncultured bone marrow cells from control animals were transplanted (Fig. 2A).

Results from transplanted mouse marrow were comparable with those observed for samples derived from rat marrow (Fig. 2B). Distinct multilineage tissue formation was consistently observed when 2–4 $\times$ 10^6 cells from 5-FU-treated animals were incorporated into implant sponges. Each of the implants had distinctly thickened cortexes and well-developed marrow

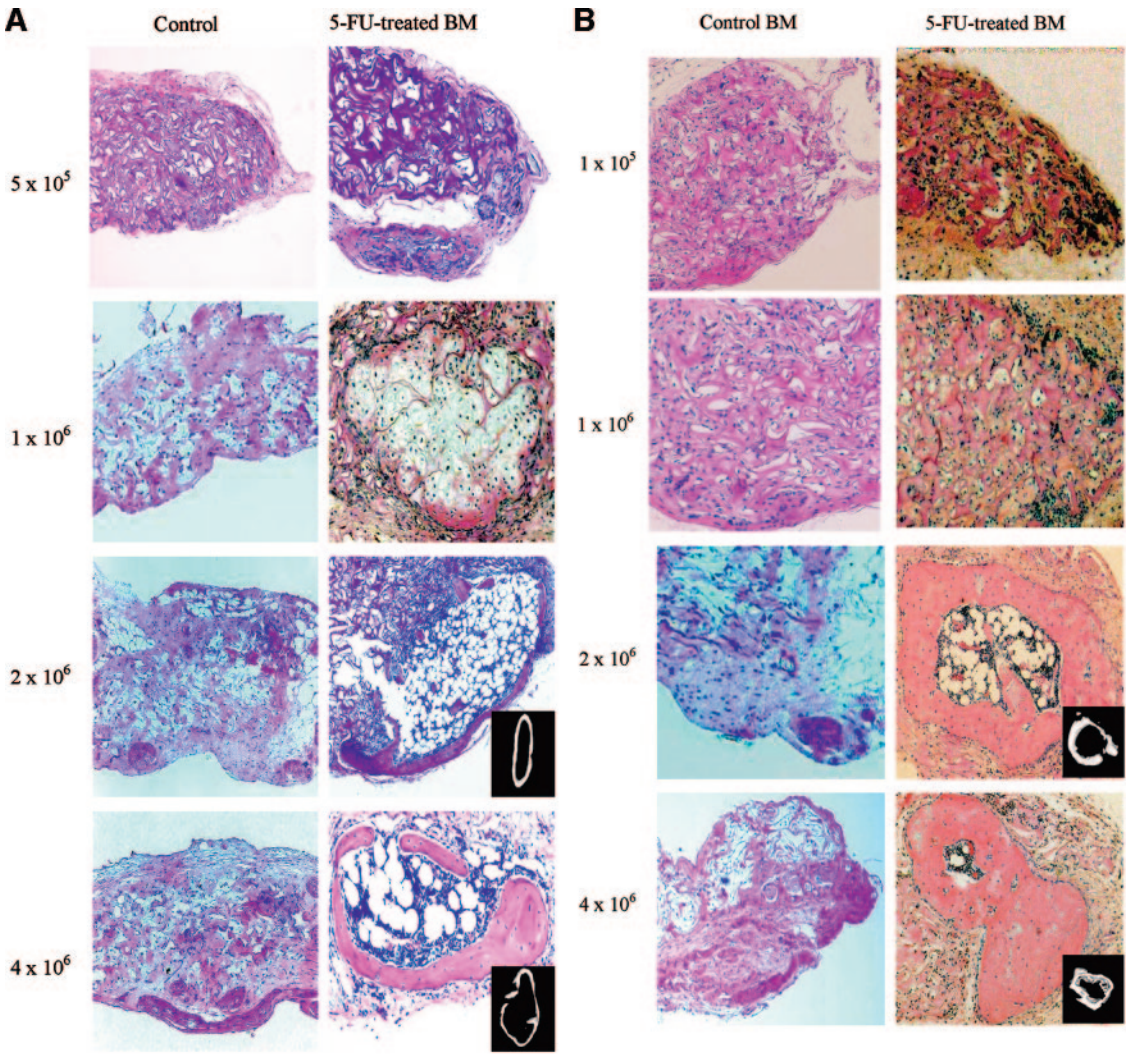

Figure 2. In vivo differentiation of bone marrow cells. Donor low-density marrow cells were isolated from rats **(A)** or mice **(B)** treated with 5-FU or vehicle. Recipient immunodeficient mice were transplanted with limiting dilutions of low-density mononuclear cells in gelatin sponges over a dose range of 0–4 $\times$ 10^6 cells/implant. After 5 weeks, the tissues were recovered, radiographed, and prepared for histology. In each case, sponges alone failed to demonstrate any organized tissue regeneration (data not presented). H&E sections presented at a magnification of $\times$100 for sponges containing 5 $\times$ 10^5 cells also failed to demonstrate distinct tissue generation. Implantation of 1.0 $\times$ 10^6 cells from the 5-FU-treated group produced hypertrophic-like chondrocytes, but the vehicle-treated group did not. At 2 $\times$ 10^6 cells/implant, the 5-FU-treated groups demonstrated significant bone deposition resulting from osteoblastic differentiation, adipocytes, and the recruitment of marrow, whereas the control did not. Further enhancement of marrow organ development including an intact cortex, medullarlary adipose tissue, and a mature marrow cavity occurred following implantation of 4 $\times$ 10^6 5-FU-treated cells, which was not seen in the vehicle-treated animals. Three-dimensional cross-sectional μCT views of implants also identified radiopaque bone in 5-FU-treated bone marrow implants at two and 4 million implants (insert). The μCT threshold was set at 1,500. Abbreviations: 5-FU, 5-fluorouracil; BM, bone marrow.

structures. At cell densities lower than 1 $\times$ 10^6 per implant, less consistent results were observed and implants were comprised of loosely organized connective tissue.

Micro-CT Images and Quantitative Analysis

Microcomputed tomographic (μCT) analysis of the recovered ossicles revealed intact bone cortexes with a well-developed marrow cavity (Fig. 2A, 2B, inserts). By comparing the bone volume of each group, there was a significantly greater volume in implants from 4 $\times$ 10^6 mouse-nucleated cells than those tissues generated by implants with 2 $\times$ 10^6 mouse cells (1.35 $\pm$ 0.03 vs. 0.35 $\pm$ 0.02 mm^3, Fig. 2B, $p < .05$). However, such differences were not observed in rat samples (0.34 $\pm$ 0.03 vs. 0.33 $\pm$ 0.01 mm^3, Fig. 2A), suggesting that the timing of cell differentiation and extent of bone mineralization are different between the two species in this model system. Quantitative analysis by μCT demonstrated significantly higher bone mineral density (BMD) values in 4 $\times$ 10^6 cell implants than that of 2 $\times$ 10^6 cell implants. This difference was consistent in both rat and mouse groups (142.26 $\pm$ 6.37 vs. 96.72 $\pm$ 22.76 mg/mm^3 in rat; 227.13 $\pm$ 54.25 vs. 128.16 $\pm$ 11.24 mg/mm^3 in mouse $p < .05$) (Fig. 3).

MTS Assays of Both Rat 5-FU-Treated Bone Marrow Cells

One explanation for the increase in CFU-F frequency and tissue generated by implanted cells is that 5-FU either spared the stem

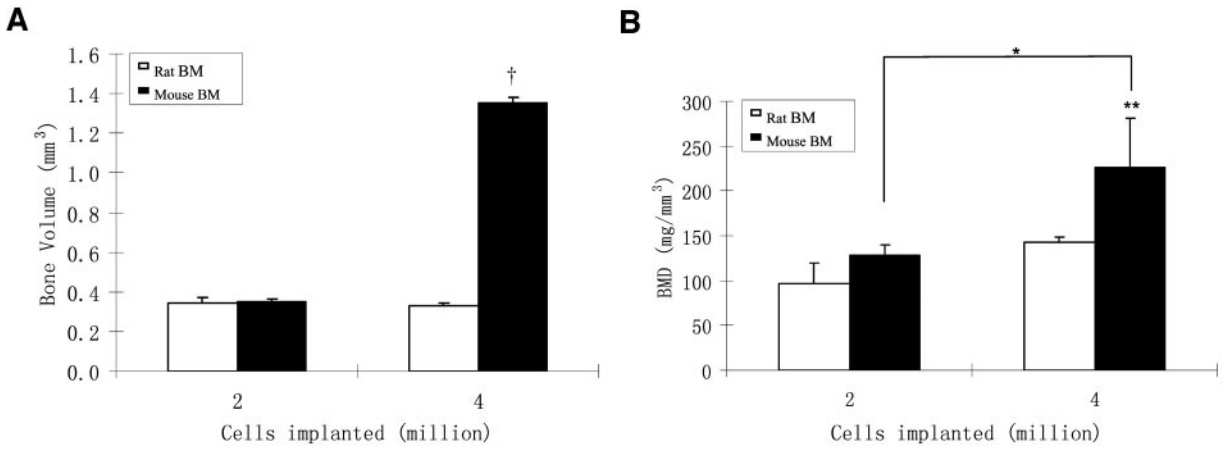

Figure 3. Quantative analysis of 5-FU-treated BM implants, measured by micro-CT. **(A):** The comparison of bone volume (mm^3) in mouse and rat BM cell implants, †, $p < .01$ at 4×10^6 in the mouse BM cell group. **(B):** The comparison of BMD in both rat and mouse cell implants. *, **, $p < .05$ for 4×10^6 mouse BM group relative to both mouse 2×10^6 and rat 4×10^6 group. Abbreviations: BM, bone marrow; BMD, bone mineral density.

cells or allowed the expansion in their numbers. As an initial attempt to explore whether the proliferative state of the 5-FU-recovered cells was different from vehicle-treated cells, proliferation assays were performed. Recovered cells were plated at low density, and proliferation was measured spectrophotometrically. In this assay, both rat and mouse BMSCs proliferated slowly when cultured in vitro (Fig. 4).

In Vitro Cell Differentiation

Another possible way that 5-FU treatment may have increased the CFU-F frequency and the complexity of the tissue generated in vivo is that the drug spared the stem cells that were in a relative quiescent state, thus increasing the frequency of stem cells per marrow cell. Should this possibility be correct, one would expect that the capacity for multilineage tissue generation would remain intact following 5-FU treatment. To evaluate this possibility in vitro, the differentiation potential of the in vivo 5-FU-treated bone marrow cells was compared with those derived from control animals cultured under adipogenic, osteogenic, or chondrogenic conditions. After 3 weeks in lineage-specific culture conditions, the expanded cells from both the 5-FU-treated and control animals were characterized. We found that lineage-specific induction was maintained for each culture condition. Adipogenic induction was apparent by the accumulation of lipid-rich vacuoles within cells (Fig. 5; Oil Red staining) following culture. Interestingly, 5-FU-treated bone marrow cells formed significant larger aggregates of mineralized nodules with von Kossa staining compared with control. Cells also showed positive staining for chondrocytic proteoglycans in both the 5-FU and control groups (Fig. 5; Safranin O staining).

Evaluation of Lineage Commitment

To determine the long-term changes of gene expression by 5-FU treatment, we cultured the cells under adipogenic, chondrogenic, and osteogenic conditions. Bone marrow treated with vehicle alone from either rat or mouse displayed an overlapping gene expression pattern when placed in lineage specific culture conditions. For example, multidifferentiation gene expression was observed for rat marrow cells placed in osteogenic or chondrogenic conditions. 5-FU-treated bone marrow cells, however, displayed a more restricted phenotype following lineage differentiation (Fig. 6A, 6B). Murine marrow treated with 5-FU demonstrated a high degree of fidelity to lineage commitment compared with the more promiscuous phenotype displayed by the untreated cells. These findings confirm our hypothesis that 5-FU spared the most undifferentiated mesenchymal stem cells in vivo but may have eliminated progenitor cells with multilineage commitment, resulting in the purification of a less committed cell.

DISCUSSION

The identification and purification of mesenchymal stem cells has been hampered by the lack of in vitro and in vivo assays that permit the rigorous identification of stem cells from mesenchymal tissues. For some time, it has been known that hemopoietic marrow is formed in bone ossicles formed by transplanted BMSCs [23, 25, 33–38]. The blood cells formed in these ossicles display multilineage characteristics by representing all the mature blood cell compartments [39]. In addition, the bone organs are fully integrated with the hematopoietic demands placed on the organism [40]. When bone matrix or purified bone-derived proteins are implanted into subdermal spaces of rodents [33, 41, 42], bone proteins induce the recruitment of pluripotent mesenchymal and circulating hematopoietic cells of host origin to form the developing organ. Recently, it has been demonstrated that syngenic donor BMSCs also form bone ossicles in a murine host [24]. Here, the mixed BMSCs establish a microenvironment that supports host-derived hematopoiesis and contains multilineaged committed cells (adipocytes, chondrocytes, osteoblasts) derived from donor cells [3, 29, 40, 41, 43]. Together, these findings indicate that BMSCs cultured in vitro maintain to some degree the potential to form multilineage tissue aggregates or marrow organs. Yet rigorous determination of the identity of MSCs has not been achieved.

Although there has been some success in expanding rodent and human BMSCs that retain the capacity for mineralization in vitro and bone formation in vivo, the problems of cellular heterogeneity and lack of predictors of success will

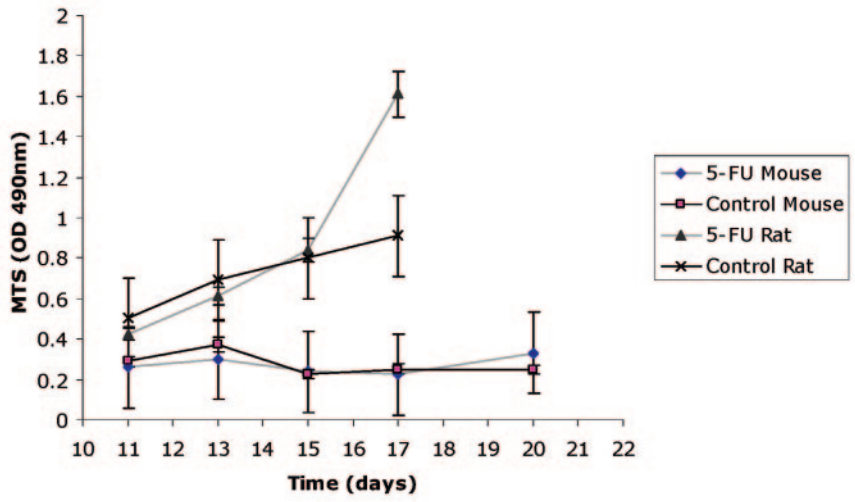

Figure 4. Cell proliferation of bone marrow stromal cells, assessed by MTS analysis. Samples were harvested on alternating days for the 1st week, and at days 17 (for rat) and 20 (for mouse) to ensure continued cell viability. No significant differences were observed between 5-FU-treated bone marrow and control ($p > .05$). Abbreviations: 5-FU, 5-fluorouracil; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) 2-(4-sulfonphenyl)-2*H*-tetrazolium; OD, optical density.

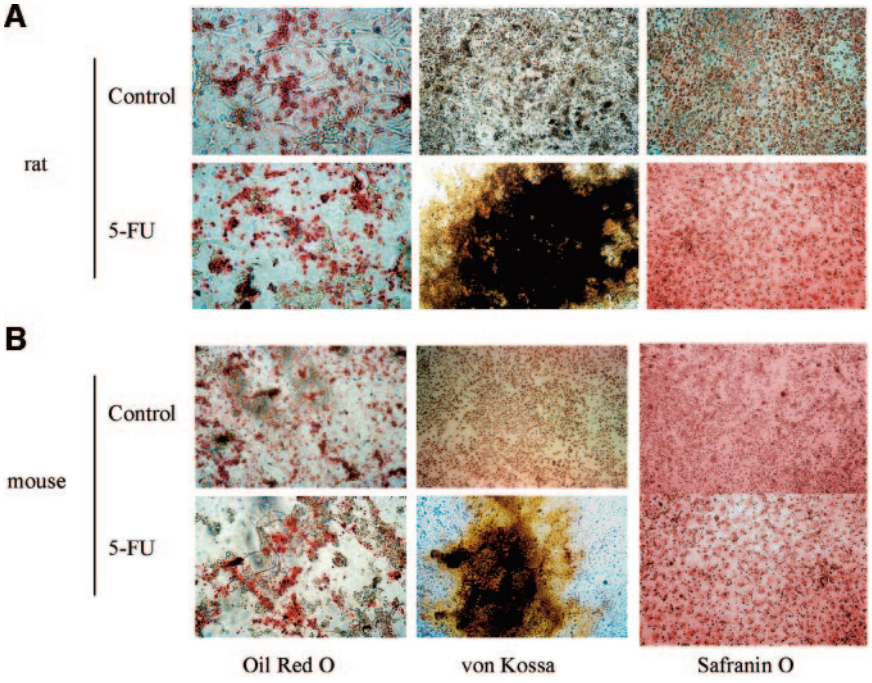

Figure 5. Comparison of the differentiation potential of normal bone marrow and 5-FU-treated bone marrow cells. Cultured bone marrow cells from control or 5-FU-treated rats **(A)** and mice **(B)** were tested for the ability to differentiate in vitro to multiple lineages at day 21 after culture in lineage specific medium. Adipogenesis, osteogenesis, and chondrogenesis were indicated by Oil Red, von Kossa, and Safranin O, respectively. Abbreviation: 5-FU, 5-fluorouracil.

ultimately impede the clinical utility of these cells. Several in vitro experimental manipulations have been performed in attempts to characterize BMSCs [3, 41, 43–50]. However, the in vitro manipulation of the cells, without a clear identification of the stem cell phenotype, limits scientific advancement or potential therapies.

The strategy of using 5-FU and low-density separation increased the frequency of CFU-F in vitro and increased the frequency of cells capable of forming bony organs in vivo without the need to expand the progenitor cells in vitro. We found that the transplantation of 2 million unfractionated nucleated cells from 5-FU-treated bone marrow generated intact ossicles containing a mineralized cortex, trabecular structure, fat, mature bone marrow, and blood vessels (Fig. 2). Based on the enrichment defined in Table 1, it is estimated that 200 to 400 CFU-F cells can form such ossicles, indicating there is a minimal requirement of cell numbers for cell interaction to constitute an appropriate microenvironment to produce bone. In comparison, non-5-FU-treated bone marrow transplantation only formed fibrous-like tissues, indicating the enrichment and purification effects of 5-FU play an important role in osteogenesis in vivo.

Several possibilities exist to account for these observations. The vast majority of available data suggests that hematopoietic and possibly mesenchymal stem cells cycle slowly such that at any given time, the vast majority of the cells are in G_0 phase of the cell cycle. The majority of stem cells would be relatively resistant to metabolic poisons or treatments that target rapidly cycling cells. In the case of a single dose of 5-FU, it is possible that the drug may trigger the release of a quiescent stem cell pool, resulting in either stem cell expansion or commitment to progenitor populations to regenerate those damaged cell

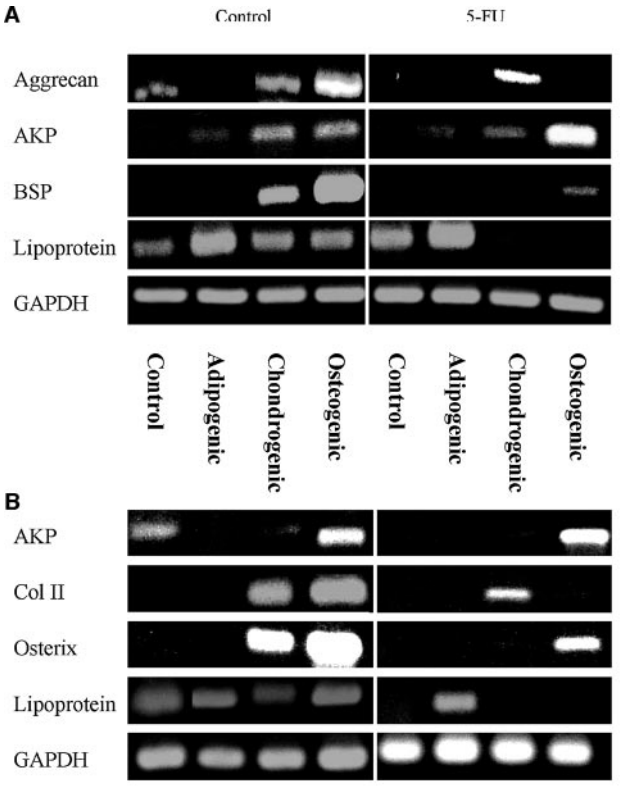

Figure 6. Gene expression by RT-PCR analysis. RNA was extracted from rat or mouse whole bone marrow 5 days after injection of 5-FU. Bone marrow from rats **(A)** or mice **(B)** was treated with 5-FU or vehicle in vivo. The bone marrow stromal cells were harvested and cultured in lineage-specific differentiating medium for 21 days. 5-FU-treated bone marrow showed greater fidelity for lineage progression. Abbreviations: 5-FU, 5-fluorouracil; AKP, alkaline phosphatase; BSP, bone sialoprotein; Col II, collagen type II; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

compartments. Alternatively, it is also possible that stem cells fail to commit to any of the lineages but rather were enriched on a per cell basis of the remaining viable cells. From our data, we cannot distinguish between these two possibilities. Previous ex vivo approaches using 5-FU-treated mouse bone marrow cells have enriched the CFU-F and osteogenic progenitor cells by as much as 12-fold and demonstrated the expression of bone-specific markers in vitro [31]. Our strategy enriched CFU-F 10-fold in rats and sixfold in mice. In vitro proliferation assays of the recovered cells indicated no significant differences between 5-FU treatment and normal BMSCs; however, these investigations may not have been sensitive enough based on purity alone to determine differences in stem cell or progenitor cell behavior.

It was recently reported that 5-FU impaired the stem cell engraftment functions depending on whether the host animals were lethally or sublethally conditioned [51]. As such, 5-FU may activate mesenchymal cells to support hematopoietic stem cell activities. We observed that 5-FU-treated bone marrow exerted an enhanced and selective effect on osteogenic differentiation as determined by von Kossa staining of BMSCs cultures in vitro compared with control BMSC cultures. However, no apparent differences were detected in adipogenic or chondrogenic differentiation effects between 5-FU and control BMSC cultures. The observation of multilineage differentiation may be due to the presence of multilineage-committed progenitor cells, indicating 5-FU functions by sparing and enriching the stem cells in bone marrow.

We found that bone marrow treated with vehicle alone from either rat or mouse displayed an overlapping gene expression pattern when placed in lineage-specific culture conditions. These data suggest that a large percentage of the progenitors were heterogeneous at the time of marrow extraction. It would be expected that a more restricted phenotype would be observed if more pure cell populations were cultured in lineage-specific conditions. This, in fact, was observed in this study, when the enriched cells displayed an enhanced fidelity to lineage commitment compared with the more promiscuous phenotype of the control cells. These findings support the hypothesis that 5-FU spared those undifferentiated mesenchymal stem cells in vivo and eliminated proliferative, lineage promiscuous cells. Thus, this work links multilineage differentiation in vivo to cell frequency in vitro in both rats and mice. As such, we propose that this assay may be useful for further isolation and ultimate characterization of noncultured mesenchymal stem cells, that is, the correlate to the SCID-repopulating assay used to identify hematopoietic stem cells.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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