Adult Stem Cell Mobilization Enhances Intramembranous Bone Regeneration

A Pilot Study

Margaret A. McNulty PhD, Amarjit S. Viridi PhD,
Kent W. Christopherson PhD, Kotaro Sena DDS, PhD,
Robin R. Frank BS, Dale R. Sumner PhD

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Abstract

Background Stem cell mobilization, which is defined as the forced egress of stem cells from the bone marrow to the peripheral blood (PB) using chemokine receptor agonists, is an emerging concept for enhancing tissue regeneration. However, the effect of stem cell mobilization by a single injection of the C-X-C chemokine receptor type 4 (CXCR4) antagonist AMD3100 on intramembranous bone regeneration is unclear.

Questions/purposes We therefore asked: Does AMD3100 mobilize adult stem cells in C57BL/6 mice? Are stem cells mobilized to the PB after marrow ablation? And does AMD3100 enhance bone regeneration?

Methods Female C57BL/6 mice underwent femoral marrow ablation surgery alone (n = 25), systemic injection of AMD3100 alone (n = 15), or surgery plus AMD3100 (n = 57). We used colony-forming unit assays, flow cytometry, and micro-CT to investigate mobilization of

mesenchymal stem cells, endothelial progenitor cells, and hematopoietic stem cells to the PB and bone regeneration. **Results** AMD3100 induced mobilization of stem cells to the PB, resulting in a 40-fold increase in mesenchymal stem cells. The marrow ablation injury mobilized all three cell types to the PB over time. Administration of AMD3100 led to a 60% increase in bone regeneration at Day 21.

Conclusions A single injection of a CXCR4 antagonist lead to stem cell mobilization and enhanced bone volume in the mouse marrow ablation model of intramembranous bone regeneration.

Clinical Relevance The emerging paradigm of mobilizing endogenous adult stem cells to stimulate tissue regeneration may lead to novel therapeutic strategies for improving repair of skeletal tissues.

Introduction

The use of autologous or allogeneic adult stem cells for tissue repair has received considerable attention. The current state of the art is to use isolated mesenchymal stem cells (MSCs) (also commonly called marrow stromal cells) or endothelial progenitor cells (EPCs) [9]. These cells are typically collected from bone marrow [2, 5, 49], muscle [5], or fat [24] through invasive procedures, often expanded ex vivo, and reimplanted at the target site.

An emerging concept is to employ endogenous stem cells mobilized to the peripheral blood (PB) [9] to achieve the same end point (Fig. 1). Osteogenic cells appear to circulate at low levels [30, 47], but the proportion of circulating osteogenic stem or progenitor cells can be enhanced through mobilization, which is defined as forced egress of stem/progenitor cells from their niche(s),

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M. A. McNulty, A. S. Viridi, K. Sena, D. R. Sumner (✉)
Department of Anatomy & Cell Biology, Rush University
Medical Center, 600 Paulina Street, Chicago, IL 60612, USA
e-mail: Rick_Sumner@rush.edu

K. W. Christopherson, R. R. Frank
Division of Hematology & Oncology, Rush University
Medical Center, Chicago, IL, USA

primarily in bone marrow, to the PB [36]. The strategy of purposefully mobilizing stem cells began with the original observation that chemotherapy increases circulating CD34⁺ cells (a marker for hematopoietic stem cells [HSCs] and progenitor cells) in patients undergoing cancer treatment [50]. This technique is considered the standard of care for adult patients who need bone marrow transplants to reconstitute hematopoiesis after chemotherapy for certain cancers [50, 51]. The ex vivo phase of expansion and in vivo placement can be avoided with mobilizing endogenous stem and progenitor cells for in situ tissue regeneration as exemplified by improved cardiac function and prolonged survival after myocardial infarction in a mouse model [25].

With respect to bone repair, this new concept is plausible because there is an abundant reserve of endogenous adult stem cells in bone marrow [21]. These endogenous progenitor cells can be mobilized to the PB [1, 11, 12, 15, 17, 22, 36, 48, 52]. Circulating MSCs, and perhaps other adult stem cells, home to sites of skeletal injury [14, 23, 29, 35, 45, 54], and increasing the number of adult stem cells at sites of injury aids tissue regeneration [4, 8, 16, 40, 43, 56] either directly or through trophic effects [5, 8, 17, 53].

Chemokines, integrins, and selectins alone or in combination maintain stem cells within their niche [33, 39] and therefore are potential pharmacologic targets for enhancing

tissue repair through mobilization. The stromal cell-derived factor 1/C-X-C chemokine receptor type 4 (SDF-1/CXCR4) axis is a particularly interesting target because of its known role in HSC mobilization [13] and its likely role in bone repair [28]. Transient antagonism of CXCR4 by the small molecule known as AMD3100 induces mobilization [48] and improves bone formation in an ectopic model [59] and in a cranial defect model [57] but has not yet been investigated in a marrow ablation model. The ablation model was chosen as a model with relevance to situations where intramembranous bone regeneration is important, such as implant fixation, distraction osteogenesis, and fracture repair using fixators.

Marrow ablation induces intramembranous bone regeneration by physically removing native bone and inducing a repair response with defined histologic phases of healing and temporal patterns of gene expression [58] and provides relatively quick end points for assessing bone repair. However, the potential effects of AMD3100-induced stem cell mobilization on intramembranous bone regeneration after marrow ablation is unclear.

We therefore posed the following questions: (1) Does AMD3100 mobilize adult stem cells in C57BL/6 mice? (2) Are stem cells mobilized to the PB after the marrow ablation procedure? And (3) is there evidence that use of AMD3100 leads to enhanced bone regeneration in the model?

Fig. 1 This schematic of the mobilization strategy shows a number of ligand/receptor pairs that may be important for maintaining stem cells in their microenvironment, including SDF-1/CXCR4. It illustrates the central concept of this study, which is stem cells can be mobilized to the PB, for example, with the CXCR4 antagonist AMD3100, and these mobilized

cells will home to sites of skeletal injury and aid bone regeneration. CXCR4 = C-X-C chemokine receptor type 4; SDF-1 = stromal cell-derived factor 1; CD = cluster of differentiation; HA = hyaluronic acid; PSGL = P-selectin glycoprotein ligand; KL = kit ligand; VLA = very late antigen; VCAM = vascular cell adhesion molecule.

Materials and Methods

To answer our three questions, we performed three experiments examining the effects of AMD3100 on mouse stem cell mobilization (Experiment 1), femoral ablation on mobilization to the PB (Experiment 2), and AMD3100 on bone regeneration after marrow ablation (Experiment 3). We used 97 10-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA), which were acclimated to Rush University's animal facility for approximately 1 week before surgery or treatment (Table 1). These studies were exploratory in nature so we had few pilot data on which to perform power analyses. All protocols were approved by our institution's animal use and care committee.

In Experiment 1, 15 10-week-old female C57BL/6 mice received a single injection of AMD3100 (5 mg/kg; $n = 7$) or saline ($n = 8$) and were sacrificed 1 hour after injection. PB was collected for colony-forming unit (CFU) assays immediately after sacrifice. In Experiment 2, 25 11-week-old female C57BL/6 mice underwent marrow ablation surgery in the distal left femur. Mice ($n = 5$ /group) were sacrificed 4, 7, 10, 14, or 21 days postoperatively. PB (approximately 450 μ L/mouse) was collected via cardiac puncture for flow cytometry analysis. In Experiment 3, 57 11-week-old female C57BL/6 mice underwent femoral bone marrow ablation surgery in the left femur. These mice received a single injection of AMD3100 (5 mg/kg) or saline 3 hours after surgery and were sacrificed after 7, 14, or 21 days ($n = 9$ –10/group). The bone regeneration was assayed by micro-CT. Contralateral (unablated) femurs from the saline group served as baseline controls.

Due to the exploratory nature of this study and few published quantitative data on bone volume in this mouse

model, we could not perform a priori power analyses. The sample size utilized in this study is based on and consistent with sample sizes used in other bone repair models [44]. After the conclusion of this study, power analyses were performed for assessing one of the key outcomes in Experiment 3, bone volume per total volume (BV/TV), assuming a power of 0.8, with $\alpha = 0.05$, and expecting a 60% difference between groups (Power and Sample Size Calculations 3.0.43; <http://biostat.mc.vanderbilt.edu/PowerSampleSize>).

AMD3100 (octahydrochloride hydrate; Sigma-Aldrich Corp, St Louis, MO, USA) was reconstituted in sterile saline for injection. AMD3100 (plerixafor) is FDA approved for use in patients in combination with granulocyte colony-stimulating factor to mobilize HSCs. The antibodies used for flow cytometry (Table 2) were from BD Biosciences (Bedford, MA, USA) (Fc BlockTM; Lineage Cocktail & Isotype; allophycocyanin [APC]-H7 rat anti-mouse CD117; APC-H7 rat IgG2b, k isotype control; v500 rat anti-mouse Ly-6A/E; v500 mouse IgG2a, k isotype control; peridinin-chlorophyll-protein complex [PerCP]-Cy5.5 rat anti-mouse Flk1; PerCP-Cy5.5 rat IgG2a, k isotype control) or eBioscience Inc (San Diego, CA, USA) (anti-mouse CD90.2 [Thy1.2] fluorescein isothiocyanate [FITC], rat IgG2b, k isotype control FITC; eFluor 450 anti-mouse CD105 [endoglin]; rat IgG2a, k isotype control eFluor 450; anti-mouse CD73 phycoerythrin [PE]; rat IgG1, k isotype control PE; anti-mouse/rat CD29 PE-Cy7; Armenian hamster IgG, isotype control PE-Cy7). For CFU-fibroblast (F) colony assays, materials were from StemCell Technologies, Inc (Vancouver, Canada) (MesenCult[®] MSC basal medium; MSC stimulatory supplements). For EPC colony assays, materials were from

Table 1. Summary of study design outlining the groups in each experiment and end point assays utilized

Experiment	Surgery	Treatment	Time (days)	Sample size/group	End point assays
1	NA	Saline	NA	8	CFUs (pooled samples with 3 technical replicates within each group)
		AMD3100	NA	7	
2	Unilateral femoral ablation	NA	4	5	Flow cytometry
			7	5	
			10	5	
			14	5	
			21	5	
3	Unilateral femoral ablation	Saline	7	9	Micro-CT
			14	9	
			21	9	
		AMD3100	7	10	
			14	10	
			21	10	

NA = not applicable; CFUs = colony-forming units.

Table 2. Antibodies used for flow cytometry

Antibody name	Alias	Fluorophore	Cells (references)
CD73		PE	MSC(+) [7, 26, 27]
CD29		PE-Cy7	MSC(+) [27, 38]
CD90.2	Thy-1.2	FITC	MSC(+) [7, 18, 21, 26, 27, 38, 42]
CD117	c-Kit	APC-H7	HSC(+) [10, 18, 21]
CD105	Endoglin	v450	MSC(+) [7, 21, 26, 27, 38, 42], EPC(−) [37]
Sca-1	Ly-6A/E	v500	HSC(+) [10, 21, 48]
VEGFR2	Flk-1	PerCP-Cy5.5	EPC(+) [21, 48, 55], MSC(−) [19, 55]
Lineage panel*		APC	HSC(−) [48], EPC(−), MSC(−)

* Recognizes CD3e, CD11b, CD45R/B220, Ly-76, Ly-6G, and Ly-6C; PE = phycoerythrin; FITC = fluorescein isothiocyanate; APC = allophycocyanin; PerCP = peridinin-chlorophyll-protein complex; MSC = mesenchymal stem cell; HSC = hematopoietic stem cell; EPC = endothelial progenitor cell.

Lonza (Walkersville, MD, USA) (EGM[®]-2 Bulletkit), Miltenyi Biotec Inc (Boston, MA, USA) (mouse VEGF), and BD Biosciences (fibronectin-coated culture dish). For HSC colonies, materials were from StemCell Technologies, Inc (human erythropoietin, mIL-3, methylcellulose), ThermoFisher Scientific (Waltham, MA, USA) (L-glutamine, Iscove's modified Dulbecco's medium), MP Biomedicals, LLC (Solon, OH, USA) (β -mercaptoethanol, bovine serum albumin fraction V), and Sigma-Aldrich Corp (transferrin, insulin).

For marrow ablation surgery, animals were prepared and underwent surgery as described previously [58]. The marrow cavity of the left femur was accessed using a 25-gauge needle, mechanically ablated, and subsequently flushed with a 30-gauge needle and 0.1 mL sterile saline. Animals were given 0.05 mg/kg of buprenorphine for analgesia for up to 2 days after surgery and were sacrificed using CO₂ narcosis and secondary cervical dislocation.

For the CFU assays, we counted whole blood with a HemaVet[®] 950FS Cell Counter (Drew Scientific, Waterbury, CT, USA) to determine populations of nucleated cells; afterwards, the red blood cells were lysed, and cells from all mice in each group were pooled. All CFU assays were performed with 35-mm plates. For CFU-Fs, 0.5×10^6 cells were plated in triplicate at 37°C, 5% CO₂, 5% O₂, 100% humidity for 7 days. Colonies were counted as colonies that formed in the culture conditions and contained more than 50 cells. For CFU-EPCs, 0.5×10^6 cells were plated in triplicate at 37°C, 5% CO₂, 5% O₂, 100% humidity for 14 days. Colonies were counted as colonies that formed in the culture conditions and contained more than 50 cells. Finally, for CFU-granulocyte-macrophage progenitors (GMs), burst-forming units-erythroids (BFU-Es), and CFU-granulocytes, erythrocytes, monocytes, megakaryocytes (GEMMs), 0.2×10^6 cells were plated in triplicate at 37°C, 5% CO₂, 5% O₂, 100% humidity

for 7 days. Colonies were counted as previously described [46].

To analyze the stem cells mobilized after marrow ablation, flow cytometry was performed on PB samples from each mouse. Immediately after collection, red blood cell lysis on the PB was performed with a hypertonic solution [46]. PB from each animal was prepared for flow cytometry as previously described [46]. 1×10^6 nucleated cells from each sample were aliquoted into separate tubes and suspended in 100 μ L flow buffer (phosphate-buffered saline + 1% bovine serum albumin), incubated with Fc Block[™] for 5 to 10 minutes, and stained with appropriate amounts of antibodies or isotype controls (Table 1). Samples were incubated with antibodies for 30 to 40 minutes in the dark at 4°C, centrifuged at 1200 rpm (328 g) for 10 minutes, and resuspended in 1% formaldehyde fixation buffer for analysis. Data collection was completed on a BD LSRII flow cytometer and data analysis performed using BD FACSDiva[™] software (Becton Dickinson, San Jose, CA, USA).

For micro-CT analysis, femurs were fixed in 10% neutral-buffered formalin and stored in 70% ethanol. The entire femur was scanned in fluid (Scanco Model 40; Scanco Medical AG, Basserdorf, Switzerland) at 55 kV, 0.3-second integration time with a 10- μ m voxel size in plane and a 10- μ m slice thickness. One of us (MAM) recorded the overall femur length and evaluated multiple trabecular parameters to assess the trabecular bone architecture and density in three regions of interest (ROIs): (1) the endocortical space, from proximal to the distal growth plate to 30% of the bone length as measured from the distal end of the bone (distal ROI; Fig. 2A), (2) the endocortical space between the 30% site and the midshaft (50% site), a region that is normally largely devoid of bone (proximal ROI; Fig. 2B), and (3) the space where the needle tracked through the center of the femur, from the distal growth plate to the 50% site (needle track ROI; Fig. 2C). The output variables included

Fig. 2A–C Two-dimensional images from micro-CT scans illustrate the three ROIs used in the analyses of bone response to the marrow ablation surgery. The regions analyzed were volumetric (areas are shown for convenience). **(A)** The distal ROI includes the endocortical space extending from the growth plate to 30% of the femur's length. **(B)** The proximal ROI includes the endocortical space extending from 30% to 50% of the femur's length. **(C)** The needle track ROI includes the space from the growth plate to 50% of the femur's length through which the needle tracked during surgery.

TV, BV, BV/TV, connectivity density, structural model index (SMI), trabecular number, trabecular thickness, trabecular spacing, apparent density, material density, and degree of anisotropy. Cortical data at the midshaft of the femur were also analyzed. The output variables included cortical bone area, total cross-sectional area, medullary space area, cortical thickness, and cortical area fraction. All variables use recently established nomenclature [6].

For Experiment 1, we did not statistically analyze the CFU data since they were generated after pooling PB within each group, meaning only one sample was available per group. These data are expressed as mean with standard error of the mean (SEM) from three technical replicates (three plates/group of each cell type were created from the pooled samples). For Experiments 2 and 3, differences in micro-CT and flow cytometry data between the different time points and/or treatment groups were analyzed using ANOVAs and post hoc Bonferroni-corrected *t*-tests. Data for these two experiments are expressed as mean with SEM from biologic replicates (different mice). We used SPSS[®] v.19 (SPSS Inc, Chicago, IL, USA) for all analyses.

Results

We observed an increase ($p = 0.004$) in nucleated cells in the PB in AMD3100-treated mice 1 hour after injection (Fig. 3A). The average number of CFU-F colonies

increased in the PB of the AMD3100-treated mice (196.5 CFUs/mL PB) compared to saline-treated mice (4.8 CFUs/mL PB) (Fig. 3B). In addition, CFU-EPC colonies were undetectable in the saline group but increased with AMD3100 treatment (59.8 CFUs/mL PB) (Fig. 3C). Lastly, the average number of colonies in CFU assays evaluating HSCs (CFU-GM [Fig. 3D], BFU-E [Fig. 3E], and CFU-GEMM [Fig. 3F]) increased with AMD3100 treatment.

For stem cell mobilization after marrow ablation, data from the mice sacrificed at 4 days were not available due to overlysing of cells, but data were available for Days 7, 10, 14, and 21. Distinct populations of $\text{lin}^{-}\text{CD73}^{+}\text{CD29}^{+}$ cells (defined as MSCs; Fig. 4A), $\text{lin}^{-}\text{ckit}^{+}\text{sca1}^{+}$ cells (defined as HSCs; Fig. 4B), and $\text{lin}^{-}\text{ckit}^{+}\text{VEGFR2}^{+}$ cells (defined as EPCs; Fig. 4C) were identified within the PB. Time influenced stem cell mobilization ($p = 0.002$ for MSCs and HSCs and $p < 0.001$ for EPCs) for all three cell populations (Fig. 4). There was an increase in MSCs at 7 days when compared to 14 and 21 days ($p = 0.018$ and 0.001 , respectively). There was an increase in HSCs at 10 and 14 days when compared to 21 days ($p = 0.002$ and 0.006 , respectively). And there was an increase in EPCs at 7 days when compared to 10 and 21 days ($p = 0.018$ and $p < 0.001$, respectively). This experiment suggests the ablation surgery alone mobilizes MSCs, HSCs, and EPCs to the PB.

AMD3100 influenced bone regeneration. In the proximal ROI, BV/TV was different over time ($p < 0.001$), with an increase ($p = 0.043$) of BV/TV with treatment (Fig. 5A). The mice treated with AMD3100 had higher ($p = 0.047$) BV/TV than those treated with saline at 21 days. Other trabecular parameters also showed differences at 21 days, including a slightly smaller TV ($p = 0.028$), a smaller SMI (indicative of a more platelike than rodlike structure, $p = 0.037$), and a higher apparent density ($p = 0.017$) (Table 3). In the proximal ROI, BV/TV tended to increase ($p = 0.088$) in mice treated with AMD3100 at 7 days. BV/TV varied as a function of time in both the distal ROI ($p < 0.001$; Fig. 5B) and needle track ROI ($p < 0.001$; Fig. 5C). Compared to baseline controls, the surgery groups had increased BV/TV in all three ROIs whether saline or AMD3100 was administered (Fig. 5). No differences were identified in the cortical analyses or in femur lengths.

Discussion

The current state of the art in using stem cells for tissue repair is that they are harvested, manipulated *ex vivo*, and then implanted locally at the site of injury. A limitation of the *ex vivo* manipulation is the loss of, or change in, cell

Fig. 3A–F Graphs show PB analyses (mean + SEM) 1 hour after a single injection of AMD3100 for (A) total nucleated cell counts and for (B) CFU-Fs (MSCs), (C) CFU-EPCs (EPCs), (D) CFU-GMs (HSCs), (E) BFU-E (HSCs), and (F) CFU-GEMM (HSCs).

surface markers, which is thought to lead to reduced engraftment efficiency. A second limitation is the expense and time involved in collecting and manipulating the cells, although there are some intraoperative techniques now becoming available for concentrating factors or cells from retrieved tissue. The stem cell mobilization strategy in regenerative medicine relies on the release of stem and progenitor cells from their niches to increase the number of circulating stem cells in the peripheral circulation. This new paradigm uses pharmacologic induction of mobilization and endogenous mechanisms of cell homing and engraftment to sites of injury. It is plausible this strategy simply mimics and amplifies normal repair mechanisms as progenitor cells are mobilized to the PB after stroke [41], vascular trauma [20], musculoskeletal trauma [31, 32], fracture [3, 34], distraction osteogenesis [34], and myocardial infarction [60]. An important pathway for stem cell mobilization is the SDF-1/CXCR4 axis and there are recent

data supporting the concept that transient disruption of this receptor-ligand complex through the CXCR4 antagonist, AMD3100, enhances bone formation in vivo [57, 59]. In this study, we assessed the utility of the mouse marrow ablation model of intramembranous bone regeneration for examining this new therapeutic strategy by posing the following questions: Does AMD3100 mobilize adult stem cells in C57BL/6 mice? Are stem cells mobilized to the peripheral blood after marrow ablation? And does AMD3100 lead to enhanced bone regeneration?

Our study is subject to several limitations. First was the low numbers of test samples in each group, especially in the experiment that identified the effect of AMD3100 on bone regeneration. This was a pilot study to determine the likelihood of finding a response and was not a study to definitively evaluate the effectiveness of AMD3100 on intramembranous bone regeneration. Based on our data, it is now possible to make a realistic estimate of the sample

Fig. 4A–C Graphs show PB cell populations (mean + SEM) after marrow ablation as defined by flow cytometry for (A) MSCs ($\text{lin}^{-}\text{CD73}^{+}\text{CD29}^{+}$), (B) HSCs ($\text{lin}^{-}\text{ckit}^{+}\text{Sca1}^{+}$), and (C) EPCs ($\text{lin}^{-}\text{VEGFR2}^{+}$).

size needed for further studies. We estimate 16 to 17 mice/group/time point would be an appropriate sample size to detect a 60% difference in mean BV/TV with a power of 0.8, which is consistent with sample sizes needed in some other bone repair models such as fracture [44]. Even though we found a difference of approximately 60% with a smaller sample size in our study, a post hoc analysis indicates the power of the experiment was on the order of 0.5. The relative young age of the mice (10 weeks) is also a potential limitation. However, mice of this age are commonly used to evaluate endogenous stem cell trafficking. Further studies in adult mice may be appropriate. It would be valuable to collect PB from the mice that underwent

Fig. 5A–C Graphs show BV/TV (mean + SEM) after marrow ablation and from contralateral saline controls (baselines; no ablation surgery) from (A) the distal ROI, (B) the proximal ROI, and (C) the needle track ROI.

ablation surgery and received AMD3100 to evaluate whether AMD3100 increases or prolongs the natural mobilization in response to injury. The surgery technique itself may elicit the maximum response of bone regeneration, making any treatment differences difficult to detect. Modifications to this surgery technique performed in our

Table 3. Micro-CT results from the proximal ROI comparing saline and AMD3100 treatment at 21 days

Variable	Saline	AMD3100	p value*
TV (mm ³)	2.399 (0.052)	2.250 (0.036)	0.028
BV (mm ³)	0.043 (0.007)	0.066 (0.009)	0.067
BV/TV	0.018 (0.003)	0.029 (0.004)	0.047
Conn.D (1/mm ³)	14.460 (3.493)	33.271 (8.688)	0.071
SMI	3.19 (0.13)	2.83 (0.10)	0.037
Tb.N (1/mm)	1.54 (0.05)	1.68 (0.08)	0.181
Tb.Th (mm)	0.040 (0.003)	0.043 (0.002)	0.388
Tb.Sp (mm)	0.656 (0.019)	0.615 (0.034)	0.317
Ap.Dens (mg HA/cc)	96.5 (4.0)	112.2 (4.3)	0.017
Mat.Dens (mg HA/cc)	827.3 (9.5)	812.5 (9.2)	0.278
DA	1.510 (0.029)	1.473 (0.051)	0.545

Values are expressed as mean, with standard error of the mean in parentheses; * independent t-tests; ROI = region of interest; TV = total volume; BV = bone volume; Conn.D = connectivity density; SMI = structural model index; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular spacing; Ap.Dens = apparent density; HA = hydroxyapatite; Mat.Dens = material density; DA = degree of anisotropy.

laboratory (using a brush to ream the marrow cavity) indicate a much more robust response to surgery is possible (data not shown), implying the technique used in this study did not maximize the inherent reparative capacity. Lastly, additional observations of stem cell mobilization after marrow ablation at earlier time points (eg, Days 0, 1, 4) would be beneficial to further evaluate the magnitude of natural mobilization due to skeletal injury alone.

The first question we addressed was whether AMD3100 mobilized stem cells in the mouse strain used in our experiments. We found AMD3100 increased CFUs indicative of MSCs, HSCs, and EPCs in the PB. An extensive study performed by Pitchford et al. [48] using BALB/c mice showed a single dose of AMD3100 led to increases in HSC and EPC colonies but not MSC colonies. This difference might be attributable to the use of more stringent criteria to define MSCs by Pitchford et al. [48] or the use of different mouse strains.

We found increases in circulating MSCs, EPCs, and HSCs after marrow ablation as defined by flow cytometry, which is consistent with data from a number of models. Alm et al. [3] found circulating plastic-adherent MSCs are more common in patients after fracture than in subjects who had not fractured. Lee et al. [35] reported an increase in circulating EPCs after tibial fracture in female BALB/c mice, peaking at Day 3 postfracture, while Laing et al. [31] report a slightly early time course in response to tibial fracture in C57BL/6 mice. We found the largest increase in EPCs at Day 7, which may indicate the true peak of EPC mobilization in our model was at an earlier time point. Increased endothelial precursor cells have also been

observed after closed tibial fracture in patients [32]. Wang et al. [57] reported a doubling in circulating HSCs at 7 days after creation of a calvarial defect in C57BL/6 male mice and this number was further increased when AMD3100 was administered.

We found AMD3100 increased BV/TV by approximately 60% at 21 days in the ROI that is normally devoid of bone in intact femurs (proximal ROI). When the BV/TV measurements were made in other ROIs (distal and needle track ROIs), BV/TV tended to increase at 7 and 21 days. The needle track ROI was included to evaluate exclusively the area of known disruption to avoid diluting an effect with native bone that may not have been adequately disrupted. While the finding at 21 days appears to be a modest increase, Wang et al. [57] showed treatment with AMD3100 for 15 continuous days starting 3 days after a critical-size cranial defect was created improved bone healing at 56 and 112 days but not at 28 days. In a recent report from our laboratory, we noted a single injection of AMD3100 enhanced bone volume in a rat model of ectopic bone formation [59]. The effect was observed at 56 days but not at 28 days, although at the earlier time point there was qualitative evidence of active bone formation in the treated animals whereas the control animals already had a relatively well-established marrow compartment. Both of these models suggest the effects of AMD3100 on bone regeneration may not be evident for several weeks and it is possible that inclusion of later time points in the marrow ablation model would show larger effects.

The existing data from our study and other studies [57, 59] document the use of the CXCR4 antagonist, AMD3100, has an effect on bone formation in at least three microenvironments: ectopic, cranial, and appendicular in this model. The concept should therefore be further explored to improve bone regeneration after trauma- or surgical-induced damage to bone. There is also evidence that skeletal injury itself induces mobilization of stem cells and that administration of AMD3100 intensifies the mobilization of stem cells. The detailed mobilization kinetics for different stem cell populations needs further investigation to determine whether mobilization of specific cell populations (eg, MSCs or EPCs as opposed to HSCs) is important for skeletal repair. Although the presumed mechanism of action of AMD3100 for enhancing bone repair involves stem cell mobilization, this has not yet been clearly established. Assuming stem cell mobilization is the mechanism of action, efforts will need to be directed toward determining which cells engraft at sites of injury and assessing their role.

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