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c-Kit Function Is Necessary for In Vitro Myogenic Differentiation of Bone Marrow Hematopoietic Cells

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ABSTRACT

In recent years, the differentiation of bone marrow cells (BMCs) into myocytes has been extensively investigated, but the findings remain inconclusive. The purpose of this study was to determine the conditions necessary to induce myogenic differentiation in short-term cultures of adult BMCs, and to identify the BMC subpopulation responsible for this phenomenon. We report that high-density cultures of murine hematopoietic BMCs gave rise to spontaneous beating cell clusters in the presence of vascular endothelial and fibroblast growth factors. These clusters originated from c-kit^{pos} cells. The formation of the clusters could be completely blocked by adding a c-kit/tyrosine kinase inhibitor, Gleevec (imatinib mesylate; Novartis International, Basel, Switzerland, http://www.novartis.com), to the culture. Cluster formation was also blunted in BMCs from c-kit-deficient ($Kit^W/Kit^{W\text{-}v}$) mice. Clustered cells expressed cardiomyocyte-specific transcription factor genes Gata-4 and Nkx2.5, sarcomeric proteins β-MHC and MLC-2v, and ANF and connexin-43. Immunostaining revealed α-sarcomeric actinin expression in more than 90% of clustered cells. Under electron microscopy, the clustered cells exhibited a sarcomeric myofiber arrangement and z-bands. This study defines the microenvironment required to achieve a reproducible in vitro model of beating, myogenic cell clusters. This model could be used to examine the mechanisms responsible for the postnatal myogenic differentiation of BMCs. Our results identify c-kit^{pos} bone marrow hematopoietic cells as the source of the myogenic clusters. STEM CELLS *2009;27:1911–1920*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow mesenchymal stem cells have been reported to adopt osteoblastic, chondrocytic, and adipocytic phenotypes in vitro [1], differentiate into myogenic phenotypes (including cardiomyocyte-like cells), and contribute to muscle and cardiac regeneration in vivo [2–4]. Treating bone marrow mesenchymal stem cells with 5-azacytidine results in the formation of myotubes connected by intercalated discs that beat synchronously in culture and assume a cardiomyocytic phenotype [5, 6]. However, this process is dependent upon a demethylating agent, which limits the usefulness of these cells for in vivo applications. Other experiments have suggested that hematopoietic bone marrow cells (BMCs) undergo myogenic differentiation when in contact with neonatal cardiomyocytes or their cellular extracts [7]. Orlic et al. isolated Lin^{neg}/c-kit^{pos} cells from adult bone marrow, injected them into an ischemic heart after a myocardial infarction, and found that the cells repopulated well-differentiated myocardium with early cardiomyocytes [8]. Unable to replicate these findings, other investigators concluded that hematopoietic BMCs do not undergo myogenic differentiation [9, 10]. A more recent study reported that bone marrow c-kit^{pos} cells expressed cardiac-specific genes *Nkx2.5* and *Gata-4* when stimulated with transforming growth factor-ß (TGF-ß), but these cells did not sustain a membrane potential, which precluded their ability to beat [11]. Based on the previous studies as well as evidence of c-kit expression in early developing murine cardiomyocytes [12], we hypothesize that, within the appropriate culture environment, c-kit^{pos} cells could be the BMC population that undergoes myogenic differentiation.

This study provides the first demonstration of the conditions under which beating cell clusters are generated from short-term cultures of total bone marrow and bone marrow c-kit^{pos} hematopoietic cells isolated from adult mice. We found

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that myogenic differentiation was dependent upon high-density cultures stimulated with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and was accelerated in the presence of the c-kit ligand, stem cell factor (SCF). The process could be blocked by imatinib mesylate (Gleevec; Novartis International, Basel, Switzerland, http://www.novartis.com), suggesting a c-kit/SCF-dependent pathway. The beating clusters adopted a sarcomere-like ultrastructural organization that allowed for spontaneous and regular beating.

Materials and Methods

Animals

C57BL/6 wild-type (WT) and $Kit^{W}/Kit^{W\text{-}v}$ mice and *C57BL/6-Tg-ACTbEGFP* enhanced *green fluorescent protein (GFP)* transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME, http://www.jax.org). *Nkx2.5-GFP* knock-in mice (established by inserting GFP with cytoplasmic expression into the *Nkx2.5* allele) were provided by Dr. R. Harvey, Victor Chang Cardiac Research Institute, Sydney, Australia. Male and female mice aged 12-14 weeks were used in all experiments. The Animal Care Committee of the Toronto General Research Institute approved all experimental procedures, which were in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals*. [13]

Isolation and Culture of Murine BMCs

WT mice ($n > 50$ mice) were sacrificed by CO_2 inhalation, and femurs and tibias were collected. For each mouse, BMCs were flushed from the bones using phosphate-buffered saline (PBS) with 2% bovine serum albumin (BSA) and 1% penicillin-streptomycin. Cell aggregations were separated by gentle pipetting, and then centrifuged for 5 minutes at 1,500 rpm and 4°C. Cells were resuspended in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) containing 10% fetal calf serum (Sigma-Aldrich), 10 ng/ml VEGF, and 5 ng/ml bFGF (human recombinant proteins; R&D Systems Inc., Minneapolis, http://www.rndsystems.com). Alternatively, control suspensions generated from the same mouse were enriched with VEGF only, bFGF only, or with no growth factors added. For each condition, cell suspensions were plated into 3 wells of a 6-well plate coated with 0.1% fibronectin (BD Biosciences, San Diego, http://www.bdbiosciences.com), at a density of 1×10^6 cells/cm^2. (Throughout "Materials and Methods," except where indicated otherwise, the number [n] of mice indicates the number of individuals from which BMC suspensions were prepared and plated into 3 wells per condition, as described here.) The cells were closely observed under phase-contrast microscopy, and the media were changed when the BMCs started to attach to the culture well (after 2 days). Nonadherent cells were centrifuged and returned to the culture wells. Thereafter, half of the media were replaced every 48 hours with gentle suctioning. To assess the effect of cell density on the cultures, cells were also plated at densities less than 5×10^5/cm^2 and 1×10^5/cm^2 ($n = 6$ mice; 2 wells per condition).

Contribution of Nonadherent Cells to Cluster Formation

Three groups of experiments were performed to investigate whether the adherent cells (mesenchymal cells) or the nonadherent cells (presumably hematopoietic cells) were the source of the beating clusters. We obtained BMC cocultures from *GFP* and *WT* mice (3 mice per group) by culturing BMCs from *GFP* and *WT* mice separately, and then switching the nonadherent cells between the two cultures on day 3 after plating. The cultures were maintained as described above. The nonadherent cells were aspirated on day 3 of culture to assess whether the remaining mesenchymal cells could form clusters under the stimulation of VEGF and bFGF. Alternatively, nonadherent cells were transferred to 1-μm inserts to assess whether the remaining mesenchymal cells could form clusters under the stimulation of the growth factors along with paracrine stimulation from the nonadherent cells.

Fluorescence Activated Cell Sorting

To compare the profiles of selected stem cell populations in fresh BMCs and nonadherent BMCs on day 3 of culture, the cells were subjected to fluorescence activated cell sorting (FACS) analysis. Fresh BMCs were isolated from femurs and tibias of *WT* mice ($n = 3$ mice), using the methods described above. Nonadherent BMCs were collected with a pipette after 3 days, and resuspended in 1% BSA in PBS. One million cells were transferred into each FACS tube in 100 μl of solution. Next, 1 μl of antibody (FITC-conjugated CD45, PE-conjugated CD31, Tie-2, Sca-1, c-kit, and flk-1 [BD Biosciences], or PDGFRß-PE and allophycocyanin-conjugated PDGFR-α [eBioscience, San Diego, http://www.ebioscience.com]) was added to each tube separately. Tubes were incubated at 4°C for 30 minutes. The cells were then washed 3 times (centrifuging and resuspending in 1% BSA/PBS), and then resuspended in 300 μl of isoflow solution (DAKO, Glostrup, Denmark, http://www.dako.com). The cells were analyzed using a FC500 flow cytometer (Beckman Coulter, Inc., Fullerton, CA, http://www.beckman.com).

Culture of Chimeric Mouse BMCs

WT mice received 9.5 Gy γ-irradiation. BMCs freshly isolated from *GFP* mice were infused through the tail veins of the irradiated mice. Five weeks after cell infusion, BMCs from the chimeric mice ($n = 5$ mice) were cultured as described above.

Isolation and Culture of c-Kit^{pos} BMCs

The preceding method allowed the crude identification of the BMC population responsible for the formation of beating cell clusters. To determine which cell surface markers were involved, we isolated c-kit^{pos} cells and performed experiments similar to those described for total BMC culture. Briefly, BMCs were prepared as described ($n = 5$ mice), and the c-kit^{pos} population was isolated using a c-kit^{pos} selection kit (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) following the manufacturer's instructions. Next, 1×10^6 c-kit-enriched or c-kit-depleted populations were plated on fibronectin-coated cell culture plates with full media, and maintained as described for total BMC culture. The purity of the c-kit isolation using this process was ~96%. These results were later confirmed using the FACS-isolated c-kit^{pos} cells (>99.6% purity of c-kit^{pos} cells) ($n = 2$ mice).

c-Kit^{pos} cells were also isolated and cultured from the bone marrow of *Nkx2.5-GFP* transgenic mice ($n = 3$ mice). Briefly, c-kit^{pos} cells were isolated using a c-kit^{pos} selection kit, and cultured as described for total BMC culture. Cells were assessed for c-kit and Nkx2.5 (GFP) expression at 3 and 14 days after culture.

Phase-Contrast Microscopy and Video Motion Recording

The cultures were closely observed and photographed. Video motion recordings were made when contractile motion occurred, usually at 14 days after the initial plating. Total numbers of clusters and beating clusters were counted with a phase-contrast microscope (Nikon TE 200 inverted; Melville, NY, http://www.nikonusa.com) with ×20 objective lenses (Orca-ER; Hamamatsu, Iwata-City, Japan, http://www.hamamatsu.com) and facilitated with a heating chamber maintained at 37°C.

Cell Cluster Patch-Clamp Action Potential

Patch-clamp recordings of membrane potential were performed 14 days after plating. Briefly, the culture media were removed and replaced with extracellular solution (pH 7.4) containing 145

Table 1. Reverse-transcription PCR primer sequences and conditions

Name	Sequence	PCR conditions	Product size
β-MHC	F: 5′-ACAGAGGAAGACAGGAAGAA-3′; R: 5′-TTGCTTTATTCTGCTTCCAC-3′	65°C for 30 seconds and 72°C for 30 cycles	240
Connexin-43	F: 5′-AGTGTTACAGCGAAAGGCAG-3′; R: 5′-TTCCT-TTGACTTCAGCCTCC-3′	65°C for 30 seconds and 72°C for 36 cycles	230
MLC-2v	F:5-GCCAAGAAGCGGATAGAAGG-3; R: 5-CTGTGGTTCAGGGCTCAGTC-3	58°C, 57°C (MLC-2v) and 60°C (ANF) respectively for 1 minute and 72°C for 2 minutes for 5 cycles; followed by 30 cycles of 94°C for 45 seconds, 50°C for 1 minute and 72°C for 2 minutes	285
ANF	F:5-TTGGCTTCCAGGCCATA-3; R: 5-AAGAGGGCAGATCTATCGGA-3		499
Gata-4	F:5-AAGACGCCAGCAGGTCCTGCTGGT-3; R: 5-CGCGGTGATTATGTCCCCATGACT-3		282
Nkx2.5	F: 5-CAGTGGAGCTGGACAAAGCC-3; R: 5-TAGCGACGGTTCTGGAACCA-3	55°C for 30 seconds, and 72°C for 30 seconds for 36 cycles	217
Myogenin	F: 5′-ACC AGG AGC CCC ACT TCT AT-3; R: 5′-CAT CAG GAC AGC CCC ACT TA-3	60°C for 1 minute 30 seconds, 72°C for 1 minute for 30 cycles	722
β-Actin	F: 5-ATCGTGGGCCGCCCTAGGCACCA-3; R: 5-TTGGCCTTAGGGTTCAGAGGGG-3	58°C for 1 minute, and 72°C for 2 minutes for 30 cycles	222

Abbreviation: PCR, polymerase chain reaction.

mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 25 mM HEPES, 28 mM glucose. Beating clusters were identified microscopically. Conventional whole-cell patch-clamp recordings were made in single beating cells (randomly selected), using a glass electrode filled with 150 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 4.0 mM K_2ATP (pH 7.35). In the current-clamp mode, the membrane potential of the cells was detected using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, http://www.moleculardevices.com).

mRNA Expression of Cardiomyocyte Markers

Intact hearts, skeletal muscle, and BMCs were obtained from *WT* mice ($n = 5$ mice). Total RNA was extracted from the whole heart and skeletal muscle tissues, as well as from clustered or nonclustered cells generated in BMC cultures (with or without VEGF and bFGF treatment, respectively). Briefly, clustered cells and nonclustered cells were collected and centrifuged, the supernatant was removed, and RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany, http://www1.qiagen.com) following the manufacturer's instructions. Total RNA was extracted from whole heart (excluding the aortic root) and skeletal muscle tissues by homogenizing the tissue in Tri-reagent (Qiagen) and then purifying with RNeasy Kit. Reverse-transcription polymerase chain reaction (RT-PCR) products were obtained using a Qiagen one-step RT-PCR kit. Briefly, 2.5 μl (100 μg) of RNA was added to the master mix containing enzyme mix, deoxynucleoside triphosphates, and the primer of interest. The reverse transcript was obtained at a single cycle of 50°C for 30 minutes. The genes of interest included cardiac transcription genes Gata-4, Nkx2.5, and atrial natriuretic factor (ANF). In addition, myocyte markers β-myosin heavy chain (MHC) and ventricular myosin light chain 2 (MLC-2v), and gap junction connexin-43 were probed. All the cDNA amplification primers used for RT-PCR (with the exception of the myogenin primer) were intron spanning. The primer sequence and PCR conditions are presented in Table 1.

Immunohistochemical Staining

To assess α-sarcomeric actinin (α-SA) expression, BMCs were cultured on coverslips ($n = 3$ mice), and day-14 clusters were fixed in acetone for 5 minutes at −20°C. Cells were washed, blocked using mouse-on-mouse block (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) for 20 minutes at room temperature (RT), and incubated overnight with mouse anti-α-SA (1:400; Sigma-Aldrich). Slides were washed and incubated with anti-mouse alexa fluor 488 (both 1:400) for 1 hour at RT.

To assess β-MHC expression in BMC clusters generated from chimeric mice ($n = 5$ mice), cells were fixed with 4% paraformaldehyde and permeabilized for 20 minutes in 0.1% Triton X-100 in PBS prior to incubating overnight with goat anti-β-MHC (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com; 1:100 in PBS containing 0.03% Triton X-100 and 1% BSA). Visualization was with anti-goat alexa fluor 637 (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com). GFP^{pos} cells were identified by green epifluorescence. All nuclei were visualized using Hoechst dye. Slides were examined under a confocal microscope (Nikon Fluoview).

To assess c-kit expression in BMCs from *Nkx2.5-GFP* mice, the $c\text{-}kit^{pos}$ and $c\text{-}kit^{neg}$ fractions were fixed in 4% paraformaldehyde at 3 and 14 days after culture. The cells were incubated with rat anti-c-kit antibody (BD Biosciences) for 1 hour at RT, washed and incubated with goat anti-rat alexa fluor 555, and then counterstained with 4′,6-diamidino-2-phenylindole. $Nkx2.5^{pos}$ cells were identified by green epifluorescence. The cells were photographed using a Zeiss fluorescent microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Transmission Electron Microscopy

Cultured cells ($n = 2$ mice; 3 wells per mouse) at day 14 were fixed in culture wells with 2% glutaraldehyde in 0.1 M phosphate buffer for 2 hours, then rinsed with buffer and postfixed with 1% osmium tetroxide in buffer for 1 hour. The fixed cells were dehydrated in a graded ethanol series, and then embedded in Spurr's epoxy resin. The area of interest was cut from the embedded culture well with a fine handsaw. Thin sections (100 nm) were cut on an RMC MT6000 ultramicrotome (RMC Products, Tucson, AZ, http://www.rmcproducts.com), placed on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. Images were taken on a Philips CM100 TEM (Philips Medical System, Andover, MA, http://www.medical.philips.com/index.html) with a Kodak Megaplus digital camera (Rochester, NY, http://www.kodak.com).

Isolation and Culture of BMCs from $Kit^W/Kit^{W\text{-}v}$ Mice

To determine whether the defect in c-kit function influenced cluster formation, we isolated and cultured BMCs from $Kit^W/Kit^{W\text{-}v}$ mice ($n = 5$ mice) as described above for total BMC culture.

Inhibition with Imatinib Mesylate

To determine whether imatinib mesylate inhibits cluster formation, total BMCs, c-kit-enriched BMCs, and c-kit-depleted BMCs were treated with imatinib mesylate (2.5 μm/L; Sigma-Aldrich)

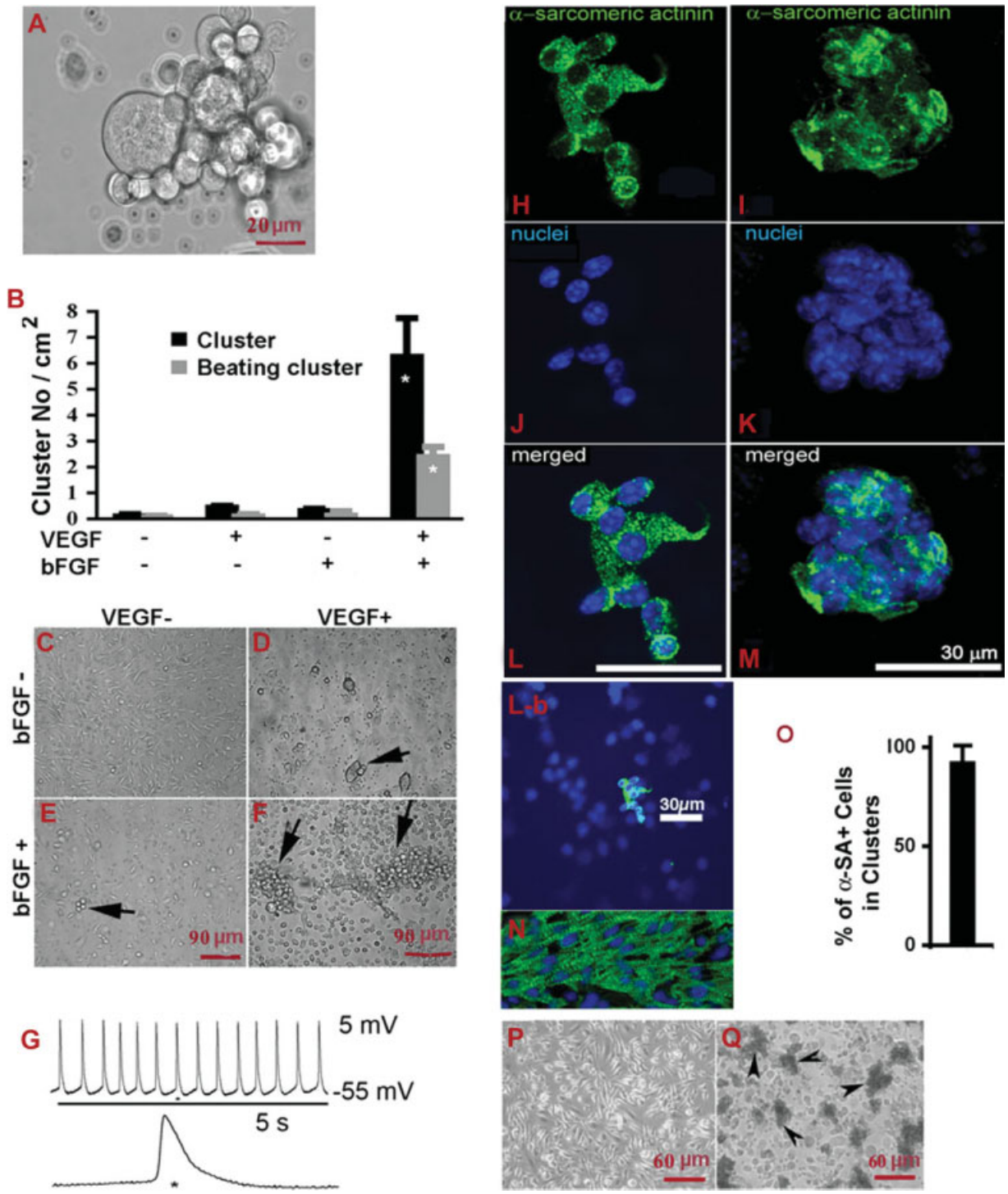

Figure 1. Characterization of BMC clusters. **(A):** Photomicrograph of a representative beating cluster after 14 days in culture. **(B):** Quantitative data showing the number of clusters formed per cm^2 in the presence of VEGF and/or bFGF (*, $p < .001$, dual treatment vs. all other groups). **(C):** In the absence of cytokines, cells assumed adherent, spindle-like morphologies with no cluster formation. **(D, E):** Cultures treated with VEGF or bFGF alone resulted in a low frequency of small cluster formation (arrows). **(F):** Cultures treated with VEGF and bFGF formed large clusters (arrows). **(G):** Plot illustrating a representative membrane action potential after patch clamping of a single clustered cell. Baseline (resting membrane potential) = −55 mV; beating rate = 168 beats per minute. **(H–K):** Immunostaining of day-14 clusters for α-sarcomeric actinin (α-SA, green). Blue = nuclear DAPI. **(L, M):** Merged images (**L–b** = lower magnification view of image in **L,** illustrating a lack of α-SA expression in nonclustered cells in the culture). **(N):** α-SA immunostaining in a representative neonatal mouse heart section, demonstrating cardiac specificity of the α-SA antibody. **(O):** More than 94% of the cells within the clusters were positive for α-SA. **(P):** Reduced initial cell density resulted in the absence of clusters in culture. **(Q):** Red blood cell colonies (arrowheads) were often observed in VEGF- and bFGF-treated cultures.

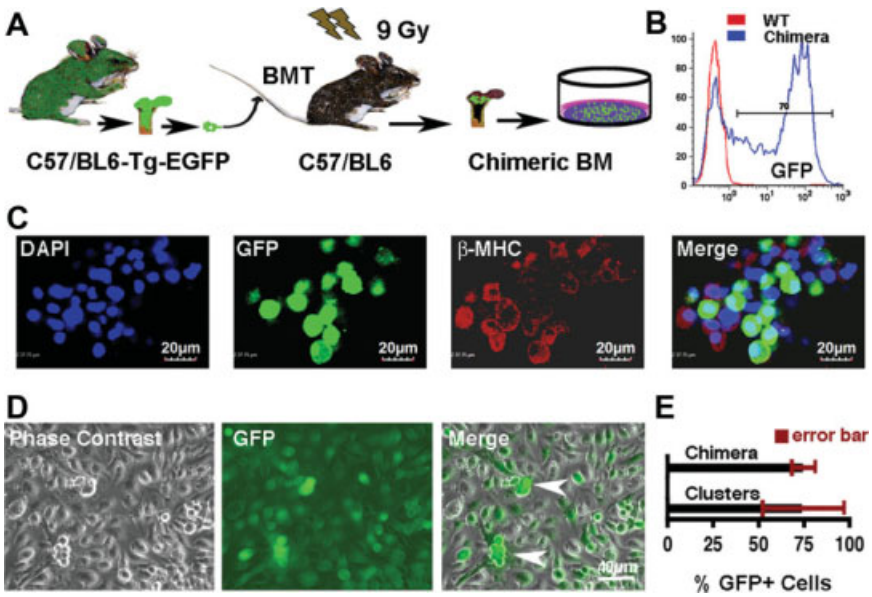

Figure 2. BMC cultures from chimeric mice. **(A):** Diagrammatic representation of the experimental strategy used to generate *GFP* bone marrow chimeric mice and BMC cultures. **(B):** Representative FACS plot illustrating the rate of reconstitution in a *GFP* chimeric mouse. **(C):** Clustered cells in cultures derived from *GFP* chimeric mice exhibited colocalization of GFP (green) and β-MHC (red) (blue = DAPI nuclear stain). **(D):** Representative phase-contrast and epifluorescent micrographs illustrating GFP expression in chimeric mouse BMCs at day 14 of culture (arrowheads = clusters). **(E):** Graph quantifying the percentage of GFP^{pos} cells among GFP chimeric mouse BMCs (Chimera), and in the clusters of beating cells that expressed β-MHC (Clusters). Abbreviation: BMT, BMC infusion; *WT*, wild-type.

in full culture media, or not treated (controls), and cultured for 3 or 14 days ($n = 3$ mice; 2 wells per condition in 48-well plates). To examine whether cell death contributes to diminished cluster formation with imatinib mesylate treatment, day-3 cultures from both imatinib mesylate-treated and control groups were analyzed by FACS for 7-amino-actinomycin D (7AAD) inclusion. Briefly, cells collected on day 3 were incubated with 7AAD for 10 minutes, and then analyzed using FC500.

Stimulation with SCF

Whole BMCs were prepared as described above ($n = 3$ mice; 24-well plates). Complete BMC media (VEGF and bFGF added) supplemented with 50 ng/ml of SCF (human recombinant protein; R&D Systems Inc.) were added to half of the plates, whereas the other half served as controls. Cells were photographed on day 14 after plating, and clusters were quantified.

Statistical Analyses

All data are presented as mean $\pm$ standard deviation. Analyses were performed using SPSS software (v. 12.0; SPSS Inc., Chicago, http://www.spss.com), with statistical significance assumed when $p < .05$. Comparisons between two groups (equal variances) were made using two-tailed paired Student's t tests. Comparisons among multiple groups were made using an analysis of variance. If the F ratio was statistically significant, then a Tukey's post hoc comparison was carried out.

Results

Myogenic Cluster Formation Requires Both VEGF and bFGF

As observed under phase-contrast microscopy, BMCs plated in media containing 10 ng/ml VEGF and 5 ng/ml bFGF at a density of $1 \times 10^6/cm^2$ began to form round cellular aggregates at 7 days after plating, and gradually formed spherical clusters semiattached to the culture dish (Fig. 1A). By days 14 to 16, approximately 25% of the clusters were contractile (supporting information Video 1; Fig. 1B). Stimulation with both VEGF and bFGF was essential for cluster formation, whereas single administration of either growth factor resulted in very a low frequency of cluster formation (Fig. 1B, 1C–1F)—indicating a synergistic effect of VEGF and bFGF. The beating clusters produced membrane action potentials typical of neonatal myocytes (Fig. 1G). Contractile proteins were localized by immunofluorescent staining of day-14 clusters, which indicated an irregular distribution of α-SA with expression in more than 94% of clustered cells (Fig. 1H–1O). α-SA was expressed by cells in all clusters surveyed (beating or nonbeating), but not by nonclustered cells in the cultures (Fig. 1L-b). Based on a yield of approximately 3-73 clustered cells (of which 94% were α-SA^{pos}) in each of 21 cell clusters counted, we estimate that myocyte yield was about 86 ± 33 myocytes per 1×10^6 BMCs plated. Additional experiments suggested that successful beating cluster formation was dependent on high-density plating, since cells plated at densities less than $5 \times 10^5/cm^2$ failed to produce clusters (Fig. 1P). In addition, several red blood cell colonies were observed within these cultures (Fig. 1Q), indicating that the culture environment permitted hematopoiesis. The beating clusters did not elongate in culture, and disintegrated within ~7 days after beating.

To confirm the origin of the beating clustered cells and to address the possibility of contaminating skeletal myoblasts, we generated BMC cultures from chimeric mice (*C57BL/6-Tg-ACTbEGFP* and *C57BL/6* [14]) with GFP^{pos} BMCs (Fig. 2A, 2B; reconstitution efficiency = 74.6% $\pm$ 3.15%). In that experiment, GFP expression by ~74% $\pm$ 22% of the myogenic (β-MHC^{pos}) clustered cells suggests that the clusters were produced from BMCs rather than from cells (such as skeletal myoblasts) introduced through external contamination during BMC preparation (Fig. 2C–2E). This likelihood was further supported by the distinctly round morphology of the clustered cells compared with the tubular structure of skeletal myoblasts [15]. These data suggested that the beating clusters formed from multiple cells rather than from single-cell colonies.

Hematopoietic, but Not Mesenchymal, Cells Are Responsible for Cluster Formation

Since the beating clusters were derived from crude BMC-derived mixtures, we attempted to determine whether the hematopoietic (nonadherent) or mesenchymal (adherent) fractions were responsible by coculturing GFP^{pos} hematopoietic cells with WT mesenchymal cells, and vice versa (Fig. 3). In both experiments, only the nonadherent cells gave rise to myogenic clusters, even after they were replated to another culture dish. The nonadherent population

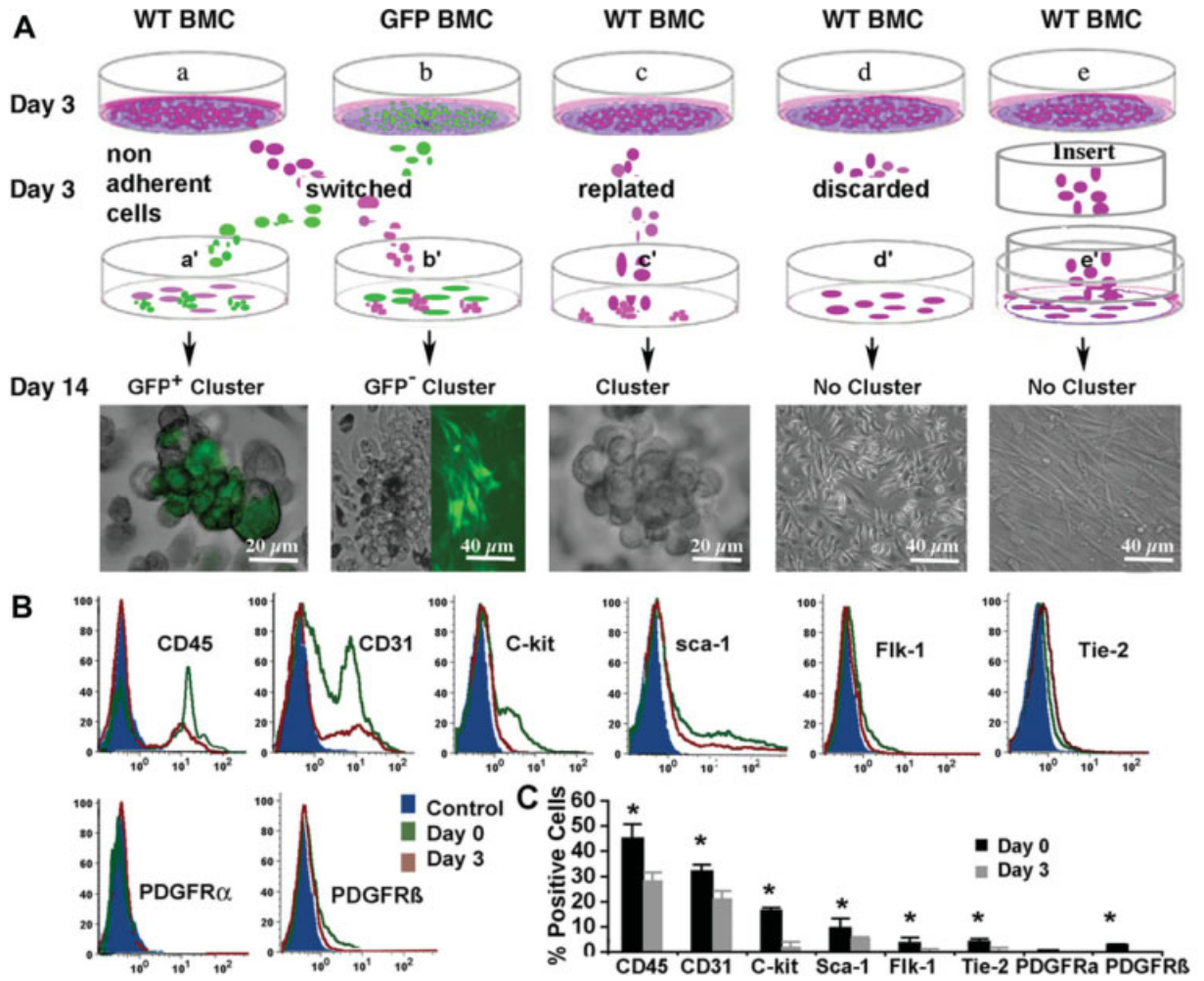

Figure 3. Contribution of hematopoietic cells to cluster formation. **(A):** Diagrammatic depiction of experimental strategy to determine cell fraction (adherent or nonadherent) responsible for cluster formation. GFP was used for cell fraction tracing. After 3 days in culture, the nonadherent (hematopoietic) cells from wild-type cultures (dish a) were removed and replated onto the GFP adherent (mesenchymal) cells (dish b'), and vice versa (dish b cells into dish a'). Photomicrographs demonstrate that the clusters were formed from the nonadherent cell fraction. Cells from dish c were replated into a new culture vessel (dish c'), suggesting that the nonadherent cell fraction did not require adherent cells for cluster formation. Accordingly, when nonadherent cells were discarded from dish d, the remaining adherent cells (dish d') showed no cluster formation, even in the presence of soluble factors from the nonadherent cell population (dish e → dish e'). **(B):** Representative FACS histograms illustrating hematopoietic markers at days 0 and 3 of culture. **(C):** Quantification of FACS analysis demonstrates the loss of hematopoietic markers at 3 days in culture: Percentages of CD45, CD31, c-kit, and Sca-1 cells were reduced significantly after culturing (*, $p < .001$, day 0 vs. day 3).

alone was therefore sufficient for beating cluster formation. Adherent cells produced no myogenic clusters, even when cultured in the presence of soluble factors released by the nonadherent cells through 1-μm membrane inserts (Fig. 3A). Together, these findings suggest that the clusters formed directly from the nonadherent hematopoietic BMC fraction.

We used FACS analysis to characterize the nonadherent BMC fraction by probing several candidate surface markers, including CD45, CD31, c-kit, Sca-1, Flk-1, Tie-2, and PDGFRs (Fig. 3B). All markers were dramatically reduced after 3 days of stimulation with VEGF and bFGF, with the largest reduction observed for c-kit (Fig. 3C). Since it is unlikely that hematopoietic cells would attach to the culture dish within 3 days, this reduction may signify the onset of myogenic differentiation.

Role of c-Kit^{pos} Cells in Cluster Formation and Myogenesis

To further characterize the hematopoietic cells that gave rise to the beating clusters, c-kit^{pos} cells were isolated and plated under conditions similar to those for total BMC cultures. Cluster formation by c-kit^{pos} cells was similar to that observed with total BMCs (Fig. 4A–4D, 4O), whereas the immunodepleted c-kit^{neg} group produced no clusters (Fig. 4E, 4F, 4O). Treatment with the tyrosine kinase inhibitor imatinib mesylate (2.5 μM/L) efficiently blocked cluster formation in both c-kit^{pos} and total BMC cultures with VEGF and bFGF (Fig. 4G–4J, 4O), suggesting that myogenic BMC differentiation could be due to tyrosine kinase receptor activation in the c-kit^{pos} cells. To test whether the cytotoxicity of the imatinib mesylate contributed to the inhibition of beating cluster formation, cell death rate was assessed by FACS analysis with 7AAD labeling. Imatinib mesylate did not reduce cluster formation via increased cell death (Fig. 4K, 4L). Furthermore, stimulation with the c-kit ligand SCF (50 ng/ml) accelerated the rate of cluster formation nearly twofold (Fig. 4M, 4N), although it did not significantly increase the number of clusters formed (Fig. 4O). Additionally, BMCs isolated from c-kit mutant ($Kit^{W}/Kit^{W\text{-}v}$) mice formed 60% fewer clusters in culture than those from *WTs* (Fig. 4P).

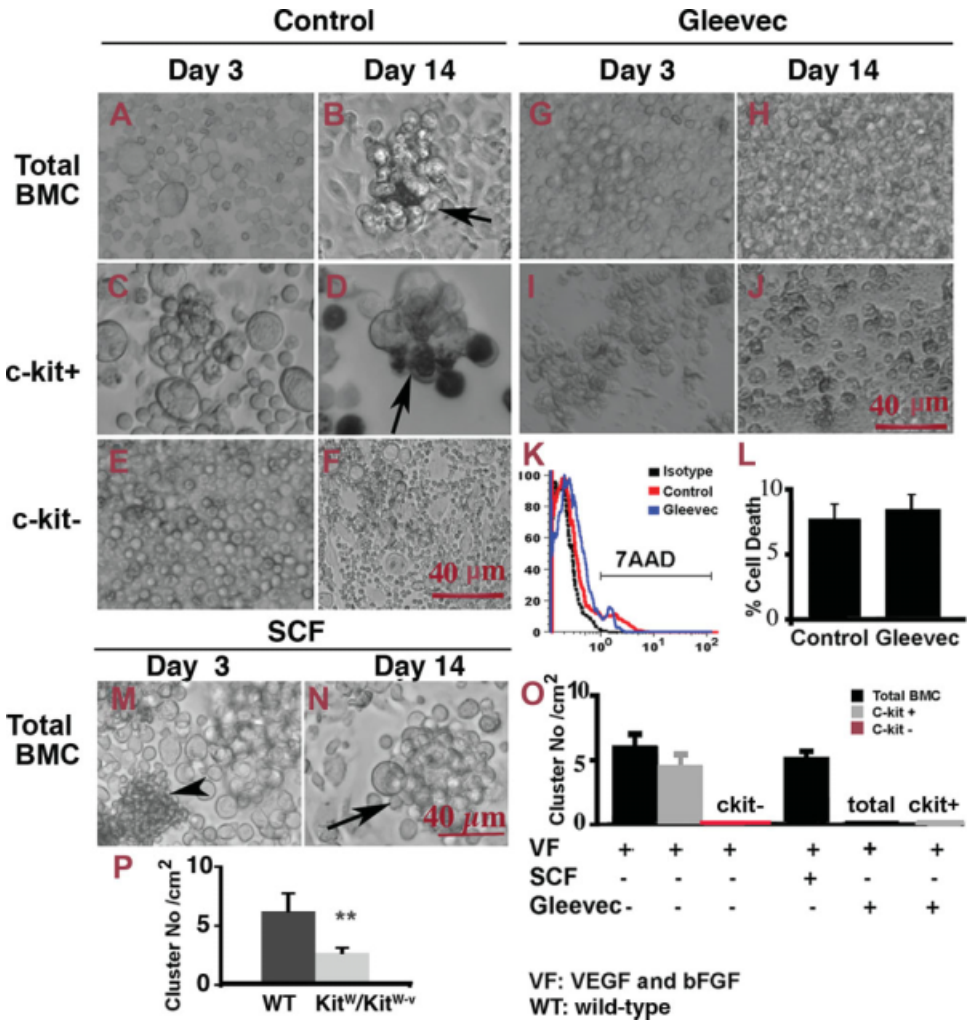

Figure 4. Role of c-kit^{pos} cells in cluster formation. **(A–D):** Photomicrographs showing total BMC and c-kit^{pos} cell cultures at day 3 and day 14 of culture. Beating cluster formation with VEGF and bFGF stimulation was observed in both cultures on day 14 (arrows). **(E, F):** c-Kit^{neg} cell culture on day 3 and day 14 failed to form clusters. **(G–J):** Imatinib mesylate treatment blocked cluster formation in both total and c-kit^{pos} cultures in the presence of VEGF and bFGF. **(K, L):** FACS plot and graph showing 7AAD-labeled cell death assay. **(M, N):** SCF treatment resulted in earlier formation of clusters, but did not increase the numbers of clusters. **(O):** Graph illustrating the number of clusters per cm^2 in the different conditions. **(P):** BMCs isolated from c-kit mutant ($Kit^W/Kit^{W\text{-}v}$) mice produced 60% fewer clusters than those from wild-type (*WT*) mice. **, $p < .01$.

To demonstrate that c-kit^{pos} cells differentiated into myogenic cells, the c-kit^{pos} fraction was isolated from the bone marrow of *Nkx2.5-GFP* mice (Fig. 5). There were no GFP^{pos} cells in the c-kit^{neg} fraction, but Nkx2.5-GFP expression was observed in 0.091% ± 0.05% of cells in the c-kit^{pos} fraction after 3 days of culture (by FACS analysis), and in 90% ± 5% of the clustered cells at 14 days (by quantifying GFP^{pos} cells in the clusters). Interestingly, some of the cells colocalized both c-kit and Nkx2.5 at 3 days—although c-kit^{pos} cells were not observed in the cultures at 7 or 14 days.

Gene Expression and Ultrastructural Features of Clustered Cells

Beating clusters and cultures without clusters were analyzed on day 14 for myogenic and cardiac mRNA expression and protein localization. Only whole heart tissue and clustered cells expressed cardiomyocyte transcription factors Gata-4 and Nkx2.5, as well as ANF, connexin-43, and genes encoding contractile proteins β-MHC and MLC-2v, but did not express the skeletal myoblast transcription factor myogenin (Fig. 6A). Accordingly, electron micrographs of the clustered cells revealed an ultrastructure reminiscent of immature myocytes (Fig. 6B, 6C) that featured numerous mitochondria and sarcomeric fibers merging at z-bands.

Discussion

This study is the first to describe the derivation of beating cell clusters from adult c-kit^{pos} BMCs, which differentiated into myogenic clusters when cultured in the presence of VEGF and bFGF. The clustered cells generated spontaneous action potentials and beat synchronously. The aggregate clusters of myogenic cells were morphologically similar to those derived from embryonic stem cells, but differed in their composition since embryoid bodies contain all three distinct germ layers.

RT-PCR analysis revealed that the clusters expressed myogenic marker β-MHC and a number of cardiomyocyte genes, including Gata-4, ANF, and Nkx2.5. Interestingly, the clusters also expressed MLC-2v, but not myogenin, suggesting a developing cardiomyocyte-like phenotype. Consistent with these data were the detection of cytoplasmic contractile protein α-SA in 94% of the clustered cells, and the presence of developing sarcomeric structures by electron microscopy. Ultrastructural sarcomeric development was characterized by the alignment of myofibers at a primitive z-disc. Taken together, our data suggest that c-kit^{pos} hematopoietic cells can differentiate into cells with characteristics that resemble immature cardiomyocytes. However, the beating clusters were not able to elongate and assume a mature, rod-like

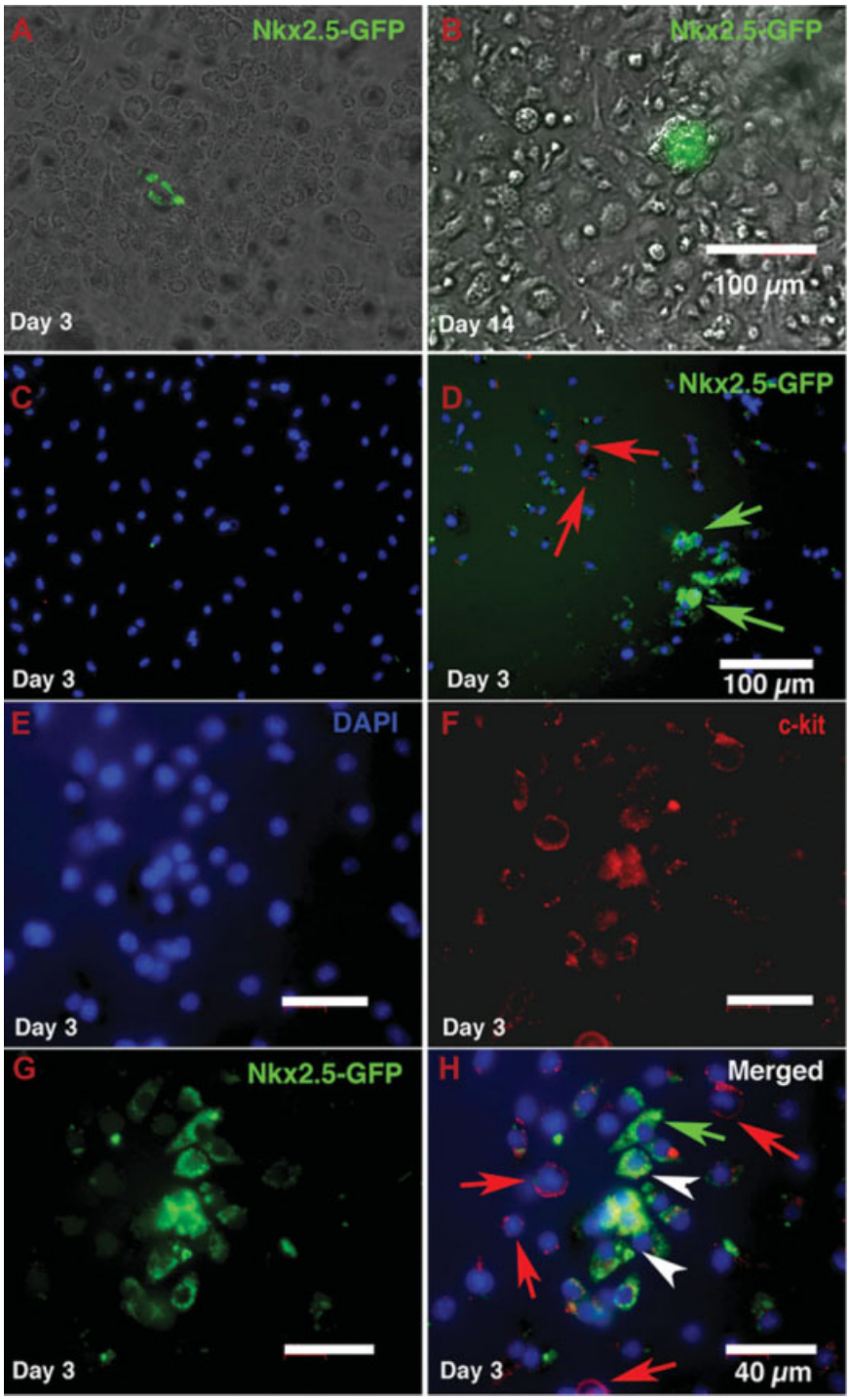

Figure 5. Nkx2.5-GFP expression in c-kit^{pos} BMCs. Representative photomicrographs (epifluorescence and phase-contrast) illustrating c-kit^{pos} cells isolated from the bone marrow of *Nkx2-5-GFP* mice. **(A, B):** Nkx2.5-GFP (green) was expressed by pockets of c-kit^{pos} BMCs at day 3 **(A)** and by cell clusters at day 14 **(B)**. **(C, D):** At day 3, c-kit and Nkx2.5-GFP were not expressed in the c-kit^{neg} fraction **(C)**, but both c-kit (red arrows) and Nkx2.5-GFP (green arrows) were expressed in the c-kit^{pos} fraction **(D)**. **(E–H):** Images at higher magnification illustrating DAPI staining (nuclei; blue, **E**) and the expression of c-kit (red, **F**, and red arrows in **H**) and Nkx2.5-GFP (green, **G**, and green arrows in **H**) at day 3 in cells from the c-kit^{pos} fraction. Merged image in **(H)** illustrates cells double-positive for c-kit and Nkx2.5-GFP (white arrowheads).

cardiomyocyte phenotype in culture. Therefore, these myogenic cells were not cardiomyocytes, but rather cells with cardiomyocyte-like characteristics.

We found that treating the cultures with both VEGF and bFGF was essential to produce beating clusters; neither VEGF nor bFGF treatment alone induced cluster formation. As a member of the BMP superfamily, bFGF is thought to be essential for renewal of the stem cell niche, and has been shown to stimulate undifferentiated murine embryonic stem cells and up-regulate the mRNA of mesodermal- and cardiac-specific transcription factor Nkx2.5 [16]. Embryoid bodies primed with bFGF demonstrate an increased propensity for cardiac differentiation, with an increase in the number of beating areas and enhanced myofibrillogenesis [16]. bFGF also contributes to cardiosphere formation in vitro [17]. VEGF, on the other hand, is thought to be important for BMC mobilization, and its effect on BMC differentiation is unclear. However, studies suggest that VEGF is a major contributor to the cardiomyogenic differentiation of adipose-derived stem cells [18]. Overexpression of the *VEGF* gene

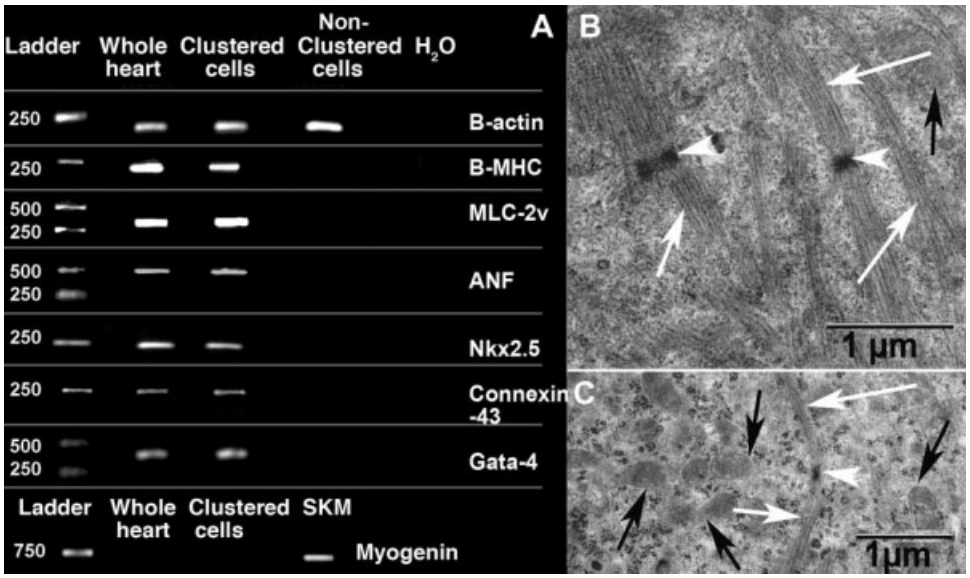

Figure 6. Gene expression in clusters by RT-PCR. **(A):** Ethidium bromide gel illustrating the following RT-PCR products in the whole heart, clustered cells or nonclustered cells: β-actin, β-MHC, MLC-2v, ANF, Nkx2.5, connexin-43, and Gata-4. Myogenin was measured in whole heart, clustered cells, and skeletal muscle (SKM). **(B, C):** Representative transmission electron photomicrographs illustrating the ultrastructure of a beating BMC cluster after 14 days in culture. **(B):** Clustered cells contained sarcomere-like fibers (white arrows) with immature z-bands (white arrowheads) and large numbers of mitochondria (black arrows). **(C):** At higher magnification, a high density of irregular sarcomeric protein accumulation and electron-dense z-bands were visible in the intracellular area.

stimulates myocytes to undergo mitosis after a myocardial infarction in sheep [19]. VEGF has also been shown to increase connexin-43 expression in cultured neonatal rat cardiomyocytes [20]. Both VEGF and bFGF act as critical factors in neonatal myocyte proliferation [21] and embryonic stem cell differentiation into cardiomyocytes [22]. It is possible that the growth factors stimulated the expression of cardiac-specific genes in the cultured BMCs.

Our studies showed that hematopoietic cells (specifically, those enriched for c-kit expression) rather than mesenchymal cells were responsible for the formation of beating clusters. The differentiation of mesenchymal stem cells into myocytic phenotypes by 5-azacytidine stimulation has been examined [5]. However, in the current study, we found that the mesenchymal population was unable to form clusters either in the presence or absence of hematopoietic cells. Conversely, c-kit-enriched cultures formed clusters without the support of a mesenchymal cell layer. Interestingly, c-kit^{pos} cells expressed cardiomyogenic marker Nkx2.5 after 3 days of culture, indicating that the c-kit^{pos} cells differentiated into myogenic cells. This finding is in agreement with reports that documented the expression of cardiomyogenic transcription factors Nkx2.5 and Gata-4 by bone marrow c-kit^{pos} cells after stimulation with TGF-ß—although those cells did not exhibit membrane potentials or beating characteristics [11]. The existence of cells double-positive for c-kit and Nkx2.5 may indicate a transient state in the differentiation of c-kit^{pos} cells into myogenic cells that is supported by a recent report of c-kit expression in early cardiomyocytes [12].

To further characterize the role of c-kit in cluster formation, we treated the cultures with tyrosine kinase inhibitor imatinib mesylate. This agent, used to treat chronic myelogenous leukemia, blocks c-kit and PDGF receptor signaling in normal cells, and BCR-ABL receptors in leukemia [23]. Imatinib mesylate has also been shown to reduce neointima formation by blocking the bone marrow-derived SCF/c-kit signaling pathway [24]. In this study, we found that imatinib mesylate treatment completely inhibited cluster formation in both total BMC- and c-kit^{pos}-enriched cell cultures, whereas SCF treatment accelerated the rate of cluster formation. In addition, BMCs isolated from c-kit-deficient mice exhibited a 60% reduction in cluster formation compared with WT BMCs. These mutant mice experience diminished cardiac function after a myocardial infarction despite a relatively normal lifespan and hematopoietic stem cell numbers [14]. c-Kit signaling therefore appears to play a major role in the formation of myogenic clusters from adult murine BMCs.

Recent studies from our group found that, although the recruitment of c-kit^{pos} BMCs is important for cardiac repair after myocardial infarction, the c-kit^{pos} cells do not themselves contribute to new myocytes [14]. The implication is that the differentiation of c-kit^{pos} cells into cardiomyocytes is not supported by the postinfarction myocardial microenvironment. Perhaps an ex vivo induction of the myogenic program followed by transplantation into the infarcted region will be necessary to achieve a functional benefit. Of course, in vivo application of the myogenic clusters will require the development of cell delivery techniques that will avoid disruption to the organized matrix structure of the heart. The cells may also be capable of coupling with residual native cardiomyocytes, as shown in other studies reporting that implanted beating embryonic or fetal cardiomyocytes [16, 25], and perhaps beating skeletal myocytes [15], can couple with residual host cardiomyocytes to impart a functional benefit.

SUMMARY

The present study describes a novel, in vitro model with which to study the molecular and cellular mechanisms associated with postnatal myogenesis in BMCs. With the improvement of transplantation techniques and the advent of cell delivery via novel biomaterials, the clustered cells may eventually be used to replace lost cardiomyocytes in vivo and restore cardiac function. In the meantime, further studies are

required to fully characterize the myogenic viability, engraftment potential, and function of the beating cell clusters.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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