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Micromarrows—Three-Dimensional Coculture of Hematopoietic Stem Cells and Mesenchymal Stromal Cells

Matthew M. Cook, B.Sc., Hons., Ph.D.,[1–3] Kathryn Futrega, B.Sc., Hons.,[1]
Michael Osiecki, B.Eng., B.Biotech., Hons.,[1] Mahboubeh Kabiri, B.Sc., M.Sc.,[1,4] Betul Kul, B.Sc.,[1]
Alison Rice, B.Sc., Hons., Ph.D.,[2,3] Kerry Atkinson, M.D.,[1–3]
Gary Brooke, B.Sc., Ph.D.,[2,3] and Michael Doran, B.Sc., B.Eng., Ph.D.[1,2]

Hematopoietic stem cell (HSC) transplant is a well established curative therapy for some hematological malignancies. However, achieving adequate supply of HSC from some donor tissues can limit both its application and ultimate efficacy. The theory that this limitation could be overcome by expanding the HSC population before transplantation has motivated numerous laboratories to develop *ex vivo* expansion processes. Pioneering work in this field utilized stromal cells as support cells in cocultures with HSC to mimic the HSC niche. We hypothesized that through translation of this classic coculture system to a three-dimensional (3D) structure we could better replicate the niche environment and in turn enhance HSC expansion. Herein we describe a novel high-throughput 3D coculture system where murine-derived HSC can be cocultured with mesenchymal stem/stromal cells (MSC) in 3D microaggregates—which we term "micromarrows." Micromarrows were formed using surface modified microwells and their ability to support HSC expansion was compared to classic two-dimensional (2D) cocultures. While both 2D and 3D systems provide only a modest total cell expansion in the minimally supplemented medium, the micromarrow system supported the expansion of approximately twice as many HSC candidates as the 2D controls. Histology revealed that at day 7, the majority of bound hematopoietic cells reside in the outer layers of the aggregate. Quantitative polymerase chain reaction demonstrates that MSC maintained in 3D aggregates express significantly higher levels of key hematopoietic niche factors relative to their 2D equivalents. Thus, we propose that the micromarrow platform represents a promising first step toward a high-throughput HSC 3D coculture system that may enable *in vitro* HSC niche recapitulation and subsequent extensive *in vitro* HSC self-renewal.

Introduction

Hematopoietic stem cell (HSC) transplantation is a curative treatment for some immunodeficiency diseases and hematological malignancies. However, inadequate supply of donor cells (especially in cord blood transplants) limits the efficacy and safety of this therapy. The hope that both efficacy and safety can be enhanced through *ex vivo* expansion of the hematopoietic populations (either the HSC themselves and/or myeloid support cells) before transfusion has motivated numerous laboratories to attempt to develop effective HSC expansion strategies. Unfortunately, strategies which enable extensive self-renewal of human HSC *in vitro* remain elusive. The current dogma is that successful self-renewal of HSC *in vitro* will require recapitulation of the bone marrow (BM) HSC niche.[1,2] The HSC niche is a complex and dynamic microenvironment containing many factors that guide HSC fate decisions. Pioneering work in this field utilized stromal cells as support cells in cocultures with HSC to mimic the HSC niche.[3] This is a logical strategy as many BM stromal cell populations have been shown to secrete or display factors which regulate hematopoiesis.[1,2]

Our understanding of the specific roles of particular stromal cell populations in the niche, and the adaption of their use in coculture, continues to evolve. Mendez-Ferrer *et al*. recently identified a subpopulation of murine mesenchymal stem/

[1]Stem Cell Therapies Laboratory, Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia.
[2]Biological Therapies Program, Mater Medical Research Institute, Brisbane, Queensland, Australia.
[3]School of Medicine, University of Queensland, Brisbane, Queensland, Australia.
[4]Department of Biotechnology, College of Science, University of Tehran, Tehran, Iran.

stromal cells (MSC) that play a critical role in supporting HSC both *in vivo* and *in vitro*.[1] Similarly, MSC subpopulations in the human system have been identified as playing critical roles in niche formation.[2] At a clinical level, Mesoblast Ltd. recently used their proprietary selected BM-derived mesenchymal progenitor cells to support the generation of a cord blood-derived hematopoietic cell product. This expanded cell product, along with unmanipulated cord blood-derived HSC, was infused into patients as part of an ongoing HSC transplant trial.[4] The combination of expanded and unmanipulated HSC populations accelerated neutrophil recovery by 14 days, and the grafts were shown to elicit less graft versus host disease relative to published outcomes using solely unmanipulated cord blood in transplants.[4]

Virtually all human HSC-MSC cocultures described in the literature, or clinically used,[5–8] have relied on a simplistic two-dimensional (2D) coculture where the HSC populations were seeded on top of a 2D stromal monolayer. An absolutely critical observation is that while these monolayer expanded cell populations have been able to confer clinical benefits, durable long-term engraftment and host repopulation from expanded HSC populations has not yet been demonstrated in humans.[4] This outcome implies that true HSC self-renewal, or even maintenance of the HSC in the starting population, has not been achieved in these culture systems. Although 2D static coculture was a logical starting point due to the operational simplicity it offers, it is likely that the monolayer culture inefficiently functions as cell–cell interactions facilitated by this geometry are inadequate.[9] By contrast, the evidence that true three-dimenstional (3D) culture results in more *in vivo* like cell behavior is persuasive,[1,10–13] and this has motivated our laboratory to develop a strategy to capture this biological potential in a 3D HSC-MSC coculture platform.

We are not alone in this respect, with recent reports revealing the specific relevance of 3D culture in the HSC field. Perhaps the most significant of these is the recent demonstration that critical niche support cells (MSC) maintain their supportive nestin expression only when cultured in 3D spheres and that this critical expression is rapidly lost when these same cells are cultured on 2D tissue culture plastic (TCP) surfaces.[1] Similarly, a few groups have now demonstrated that the presentation of the supportive stromal cell population in a 3D bioscaffold, rather than on a 2D surface, results in a significant enhancement of the coculture's potency,[14–16] and even improved *in vivo* repopulating ability.[16] While initially promising, most scaffold systems do not offer a truly 3D culture environment as the support cells are themselves attached to the biomaterial scaffold which, over the length of a single cell, is essentially 2D. Thus, while such systems no doubt enhance cell–cell interactions, the attachment of cells to a biomaterial surface may illicit the same artificial 2D cell polarity known to perturb cell function.[9] In many cases the most effective way to eliminate such artificial biomaterial interactions, maximize cell–cell interactions and to achieve *in vivo* like cell behavior is through the formation of cell aggregates.[9,17] Such scaffold-free 3D cocultures designed for the expansion of HSC populations have yet to be described in the literature, with only two studies using this type of culture system to assess HSC migration.[18,19] The most recent study utilized a nonadherent surface in a 96-well plate to encourage the self-assembly of MSC spheroids, which in a subsequent step were brought into contact with $CD34^+$ cells to study their interaction with, and migration into, the MSC aggregates.[18] Although useful for studying cellular interactions, these systems lack the efficiency necessary to practically investigate cell expansion and HSC self-renewal. A second system that has been utilized to generate cell spheroids is the hanging-drop method,[20] although this system is yet to be reported in the context of hematopoietic cell culture. A limitation of both these methods is that they are only able to produce a single aggregate of cells per well which ultimately limits the practical scalability of the systems and even limits the practicality of doing some bench-scale studies, such as the one described in this paper.

Herein we describe a novel high-throughput 3D microaggregation platform that enables the establishment of coculture spheroids of murine hematopoietic stem/progenitor cell (HSPC) populations ($Lineage^{dim}$, $Sca\text{-}1^+$ and $c\text{-}kit^+$ [LSK]) and MSC which we term "micromarrows." We then contrast the ability of the 3D micromarrow system to support HSPC expansion against conventional 2D coculture. To form our micromarrow aggregates, we have utilized the commercially available AggreWell product (StemCell Technologies). We show that cells attach and spread on the surface of this product when used with our culture system and describe a necessary surface modification step to prevent this undesirable outcome. This surface modification step limits protein adsorption and subsequent cell attachment on the AggreWell product resulting in the rapid self-assembly of unbound 3D micromarrows. In this study, we were particularly interested in assessing the benefits associated with the enhanced cell–cell interaction offered by the micromarrow system. Thus, we did not supplement HSPC cultures with cytokines other than those present in the fetal bovine serum (FBS). Overall we show that the micromarrow system enhances LSK expansion relative to 2D cultures. This platform represents a significant advancement over existing HSC coculture systems and provides a platform for synthetic niche recapitulation.

Materials and Methods

Mice

Six- to 8-week-old female C57BL/6 mice (purchased from the Australian Animal Resource Centre) or inbred C57BL/6 transgenic for green fluorescent protein (GFP) under the control of the ubiquitin promoter (C57BL/6-GFP) were used in this study. All animal experiments were approved by the University of Queensland Animal Ethics Committee.

Flow cytometry

Cell sorting and immunophenotype analysis was performed by flow cytometry using fluorochrome-labeled rat-anti mouse monoclonal antibodies (all at 1–2.5 μg/mL) as follows: c-kit allophycocyanin (APC; IgG2b; BD Bioscience), Sca-1 phycoerythrin cyanine-7 (PE Cy7; IgG2a; BD Bioscience), CD45 APC (IgG2b; BD Bioscience), CD31 PE (IgG2a; BD Bioscience), CD44 PE (IgG2b; BD Bioscience), CD11b PE (IgG2b; BD Bioscience). A biotinylated lineage cocktail (containing CD5 [IgG2a; BD Bioscience], CD45R/B220 [IgG2a; BD Bioscience], Gr-1 [IgG2b; BD Bioscience], and F4/80 [IgG2a; eBioscience]) was also used for staining of hematopoietic cells with streptavidin Pacific Blue (Invitrogen) secondary staining. When immunophenotyping was

performed, dead cells were identified by 7-aminoactinomycin D incorporation (<2% of analyzed cell populations were identified as nonviable (data not shown) and were excluded from further flow cytometric analysis. Cell sorting was performed on a FACS-ARIA (BD Bioscience) and immunophenotype was performed on a LSRII (BD Bioscience) with results analyzed in FlowJo Version 7.5 (Tree Star).

Isolation of murine HSPC and MSC populations

C57BL/6 or C57BL/6-GFP mice were sacrificed by asphyxiation and cervical dislocation. The pelvis, tibia, and femur were removed and cleaned of muscle. The bones were crushed in a mortar and pestle with the bone fragments collected and used for the isolation of MSC outlined below. The supernatant, containing BM cells, was collected for isolation of hematopoietic populations. For HSPC population isolation, c-kit$^+$ cells were first purified using CD117 conjugated magnetic beads (Miltenyi Biotec) and positive selection. The c-kit positive fraction was then stained with a biotinylated lineage cocktail in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich). Cells were then stained with c-kit APC, Sca-1 PE Cy7, and streptavidin Pacific Blue (Sigma-Aldrich) and flow sorted for Sca-1$^+$, c-kit$^+$ and lineage$^{-/low}$(LSK).

For isolation of MSC, the bone fragments from the crush were digested using 500 U/mL collagenase-I (Worthington) in Dulbecco's modified Eagle's medium (DMEM; SAFC) at 37°C on a shaker at 220 rpm for 20 min. Digested material was washed and passed through a 40 μm cell strainer to remove bone fragments and centrifuged on a Lympholyte-M (Cedarlane Laboratories) density gradient at 1500 *g* for 20 min at room temperature. The mononuclear cells were collected, washed, and plated in MSC tissue culture medium consisting of α-MEM (SAFC Biosciences) supplemented with 20% FBS (Gibco) and gentamicin (50 mg/mL) (Pfizer). Nonadherent cells were washed off after 24 h and the media was replaced twice weekly and incubated at 37°C and 5% CO_2. When 80%–90% confluent, the adherent cells were detached with TrypLE Select (Invitrogen) and stained with Sca-1 PE Cy7 and CD45 APC in PBS containing 1% BSA. Harvested cells were then flow sorted for Sca-1$^+$ and CD45$^-$. The cells were then returned to culture and further expanded. This was done by passaging the MSC when 80%–90% confluent and replating at a density of $1–3\times10^3$ cells/cm^2. All experiments were performed at passage 8–12.

Mesodermal differentiation assay

MSC were induced into adipocyte, osteoblast, and chondrocyte lineages as follows. All reagents were from Sigma-Aldrich unless otherwise specified. For adipogenic differentiation MSC were seeded in a 24-well plate and cultured in DMEM containing 3-isobutyl-1-methylxanthine (0.5 mM), indomethacin (60 μM), insulin (10 μg/mL), dexamethasone (1 μM), 10% FBS, and gentamycin (40 μg/mL; Pfizer) for 21 days. Cells were then fixed in 4% paraformaldehyde (PFA) and the presence of lipids was analyzed with Oil Red-O staining. For osteogenic differentiation, MSC were seeded in 24-well plates, grown to confluence, and cultured for 21 days in DMEM supplemented with dexamethasone (0.1 μM), β-glycerol phosphate (100 mM), L-ascorbate-2-phosphate (10 mM), calcium chloride (4 mM), 10% FBS, and gentamycin (40 μg/mL). Cells were fixed in 4% PFA and stained for the presence of calcium with Alizarin Red S solution. Chondrogenic differentiation was performed by pelletting MSC and culturing them for 21 days in DMEM, dexamethasone (0.1 μM), L-ascorbate-2-phosphate (2 mM), sodium pyruvate (1 mM), proline (40 μg/mL), transforming growth factor-β1 (10 ng/mL; Peprotech), insulin/transferrin/selenium (50 μg/mL; BD Bioscience), and gentamycin (40 μg/mL). Pellets were fixed in 4% PFA, sectioned, and stained for the presence of glycosamine glycans with Alcian Blue staining. In all instances differentiation media were changed twice per week and all samples were analyzed by light microscopy.

Surface modification

Scaffold-free 3D cocultures were established in AggreWell 24-well plates (StemCell Technologies). The AggreWell surface was modified to make it more resistant to protein adsorption and thus not compromise cell aggregate formation even in the presence of media containing FBS. The AggreWell silicone inserts were first etched by treating them with 5 M NaOH for 30 min at room temperature.[21] Wells and inserts were repeatedly washed with PBS to eliminate the NaOH. In addition, AggreWell plates were spun at 4000 *g* for 5 min with 1 mL of PBS in each well to force NaOH potentially trapped under the insert out into the PBS solution. After molecular etching with NaOH, we used electrostatic deposition to construct chitosan (CHI) and hyaluronic acid (HA) multilayers (ML) on top of the silicone inserts. The HA coated surface formed using this method has been previously shown to resist most protein adsorption.[22] In brief, the protocol used to construct the HA/CHI ML was as follows with all steps performed at room temperature. Layer-by-layer deposition of high molecular weight HA (1.78 MDa) (Lifecore Biomedical) and CHI (Wako Chemicals) was carried out in 5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer at pH 5.5. After the wash step the NaOH etched surfaces were first coated with 10 μg/mL polylysine (Sigma) that was permitted to adsorb to the surface for 30 min leaving free amine groups for subsequent cross-linking. Conversely polylysine can be just directly adsorbed to TCP controls. The surfaces were then washed twice with MES buffer. Next, 0.5 mL of freshly prepared HA solution containing 50 μg/mL HA in MES (pH 5.5) plus 1:100 cross-linker stock composed of 70 mg/mL N-hydroxysuccinimide (NHS) and 50 mg/mL *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; cross-linker) in dimethyl sulfoxide (Sigma-Aldrich) was added to each well. The electronegative HA is adsorbed to the electropositive polylysine where the NHS/EDAC facilitates the cross-linking of acid groups from the HA to amine groups on the polylysine. After 15 min incubation the surfaces were washed twice with MES buffer. Next, a layer of CHI was generated by allowing electropositive CHI from 0.5 mL of 50 μg/mL CHI in MES (5 mM, pH 5.5) to directly adsorb to the HA coated surfaces. After 15 min incubation the surfaces were washed twice with MES buffer. The repeat deposition of HA and CHI was continued until five layers of CHI were generated with the final layer being HA. The final top layer of HA was permitted to incubate overnight. Wells were rinsed three times with 0.5 mL of PBS and then incubated at 4°C for 24 h with 0.5 mL of PBS before being used in cell culture. This surface is

here after referred to as a ML. This surface modification technique and the functionality of the nonadhesive surface was validated in this study using the adherent fibroblast cell line NIH-3T3. The size of aggregates formed on ML surfaces was quantified in ImageJ (National Institutes of Health) using Feret diameter (built in function in ImageJ). Previous work, including a publication from our own team, describes the properties of this surface in more detail and quantifies its ability to resist protein adsorption.[22,23]

Coculture assay

Three-dimensional cocultures were initiated by adding 6×10^3 LSK and 1.2×10^5 MSC in 1 mL of BioWhittaker X-vivo 10 medium (Lonza) supplemented with 20% FBS and gentamycin (40 μg/mL) to each well of the AggreWell plate. The plate was then centrifuged at 400 g for 5 min causing LSK and MSC to form microaggregates at a ratio of 5 LSK to 100 MSC per microwell. Two-dimensional cocultures were concurrently initiated with the 3D cultures. This was performed by culturing 6×10^3 LSK and 1.2×10^5 MSC to each well of an unmodified 24-well plate. Both the microaggregates and 2D cultures were grown for 7 days (37°C, 5% CO_2). After the culture period, cells were harvested by vigorously flushing PBS over the patterned surface. The cells were then centrifuged (350 *g*, 5 min), resuspended in 1 mL of cell dissociation buffer (Gibco, Invitrogen), and incubated for 5 min at 37°C. After mixing, the suspension was centrifuged (350 *g*, 5 min) and resuspended in an appropriate volume of PBS supplemented with 2% FBS for cell counting and analysis of LSK markers.

RNA extraction and polymerase chain reaction

RNA was extracted with a Qiagen RNeasy Mini Kit (Qiagen) using the manufacturer's protocol. RNA was preincubated with DNase I (Invitrogen) and reverse transcription was performed with oligo-dT and Superscript III (Invitrogen) as described in the manufacturer's instructions. Quantitative real-time (qRT)-polymerase chain reaction (PCR) was performed on cDNA using ABsolutet™ QPCR SYBR® Green (ABgene). Product size and primer sequences used were as per Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/tec).

Immunofluorescence

For immunofluorescence, cultures were initiated with C57BL/6-GFP-derived MSC and C57BL/6 LSK. After the 7 day coculture period, aggregates were fixed in 4% PFA in the AggreWell Plate for 30 min at room temperature. Cultures were blocked and stained with rat anti-mouse CD45 (BD Bioscience) overnight at 4°C. They were then washed and incubated with goat anti-rat Alexa 555 (Invitrogen) at room temperature for 30 min. After washing, cultures were blotted to remove excess liquid and incubated in ProLong Gold antifade reagent mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) overnight at 4°C in the absence of light. Cultures were imaged using a Zeiss LSM510 META or Leica TCS SP5 confocal microscope.

Statistics

Mann–Whitney test was used to verify statistical significance between groups. This was performed using GraphPad Prism version 4.03 for Windows (GraphPad Software). Results were deemed statistically different if $p<0.01$. All continuous data was expressed as mean ± standard deviation. All experiments had a minimum of $n=4$ and were repeated at least three times.

Results

Characterization of the ML cell culture surface

ML surfaces on conventional TCP and inserts from the AggreWell plate were assessed for cell adhesion using the fibroblast cell line NIH-3T3. Cells were seeded into both ML and unmodified TCP surfaces and cells permitted to adhere overnight. As expected, 3T3 fibroblasts were seen to adhere to unmodified TCP surfaces (Fig. 1a). Interestingly, cellular adherence was also seen to occur on the unmodified AggreWell inserts (Fig. 1B) to a level essentially equivalent to TCP. However, cellular attachment was completely blocked on either the TCP or the AggreWell surfaces modified with the ML (Fig. 1C, D). Further, the fibroblasts were seen to spontaneously form aggregates on both ML surfaces (Fig. 1C, D). Importantly, in the context of this study, the aggregates formed on the ML TCP surfaces were heterogeneous in size ranging from 10 to 140 μm (Fig. 1E). By contrast, the aggregates formed using the ML AggreWell inserts were uniform in size with greater than 90% of the aggregates ranging from 60 to 80 μm in diameter (Fig. 1E). These results were consistent to previous published results from our group[23] and others.[22]

Isolation of murine LSK & MSC

Murine LSK were isolated from BM of C57BL/6 via magnetic cell sorting and fluorescent cell sorting (FACS). Approximately 0.001% of BM cells were LSK and had a lymphocyte-like morphology (Supplementary Fig. S1A, B). LSK were enriched for true HSC and could reconstitute the hematopoietic system, and enable 100% survival, in lethally myeloablated syngeneic recipient mice after total body irradiation (Supplementary Fig. S1C).

MSC were isolated from the hind limb bones of C57BL/6 mice via collagenase digestion of the bones followed by subsequent adherence and expansion on TCP. These cells were then purified for the Sca-1^+ $CD45^-$ fraction at the first passage by FACS. This purified population was further expanded for 8–12 passages before use. The enriched cells were plastic-adherent and exhibited classical fibroblastic morphology (Supplementary Fig. S2A). Cytospin preparations of suspensions of these undifferentiated MSC showed them to be large cells with dense chromatin, eccentric nuclei, and extensive cytoplasm full of granules (Supplementary Fig. S2B). MSC were Sca-1^+, $CD44^+$, $CD45^-$ and were devoid of endothelial cell and macrophage markers ($CD31^-$ and $CD11b^-$ respectively) (Supplementary Fig. S2C). MSC also displayed adipogenic, osteogenic, and chondrogenic differentiation as shown by histology studies (Supplementary Fig. S2D).

2D and 3D cocultures lead to hematopoietic cell expansion

The AggreWell plates are designed such that there are ~600 microwells per cm^2. Cultures were first observed by light microscopy after centrifugation to ensure aggregates were evenly distributed (Fig. 2A). As before, when aggregates

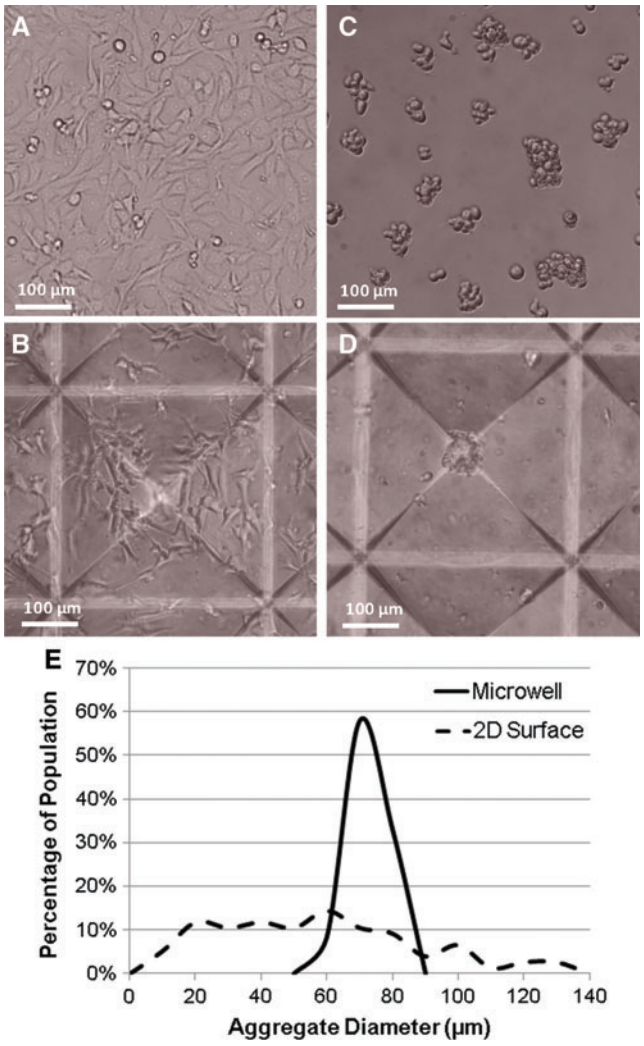

FIG. 1. Assessment of cellular attachment on multilayered (ML) surfaces. The fibroblast cell line NIH-3T3 were seeded onto tissue culture plastic (TCP) and AggreWell plates which were either unmodified or ML to prevent cell attachment and left for 24 h. The fibroblasts were seen to attach to both **(A)** unmodified TCP and **(B)** unmodified AggreWell inserts. Surface modification by the addition of a chitosan-hyaluronic acid multilayer prevented fibroblast attachment to both **(C)** TCP and **(D)** the AggreWell inserts. **(E)** Quantification of the aggregate diameters formed on these surfaces shows that aggregates formed on ML TCP are heterogeneous, having a large size distribution, whereas those formed by the AggreWell inserts are uniform. All images are by phase contrast microscopy and ×10 magnification. Color images available online at www.liebertonline.com/tec

were formed without surface modification, serum proteins enabled cell attachment and spreading on the AggreWell surface (Fig. 2B). By contrast, on the ML surface modified plates LSK and MSC self-assembled into aggregates of ~50–100 μm in diameter within 24-h, thus demonstrating a preference for cell-cell affinity rather than cell adhesion to the plate surface (Fig. 2C). Further, incorporation of LSK into the aggregates appears to be 100% as no single cells could be observed outside the microaggregates. LSK expansion cultures were simultaneously initiated on monolayers of MSC (Fig. 2D) to allow direct comparison between the different culture geometries. Apparent proliferation in the 3D cultures was prominent after 5 days of culture with small hematopoietic-like cells beginning to fill the microwells although this was not observed for all aggregates (Fig. 2E). This stochastic distribution likely reflects the fact that each aggregate contains ~5 LSK and that the LSK population is itself heterogeneous with approximately only 1 in 10 being a true HSC.[24,25] Likewise, hematopoietic proliferation could be observed in the 2D adherent cultures where the small hematopoietic cells exist above the monolayers of MSC (Fig. 2F). A further increase in cellularity was evident at day 7 of culture (Fig. 2G). Micromarrows were varied in size with some not showing any or only beginning to show visible proliferation, while in other wells the micromarrow aggregate was buried under detached hematopoietic cells. Conversely, in the 2D condition, hematopoietic cells were dispersed throughout the entire culture well (Fig. 2H).

Total cell expansion was similar between 2D and 3D cocultures

After the 7 day coculture period, the micromarrows were dispersed into a single cell suspension and total cell expansion was calculated. This revealed a trend toward a greater number of total cells in 3D LSK-MSC cocultures ($9.2 \pm 2.0 \times 10^5$) when compared with equivalent 2D controls ($7.2 \pm 0.7 \times 10^5$). This data represents a total fold expansion of 3D (150 ± 33-fold) when compared with the 2D control (120 ± 12-fold) (Fig. 3A).

A higher proportion and number of LSK were generated in 3D cocultures

The proportion of HSPC candidates was determined by flow cytometry for the LSK marker phenotype (Fig. 3B) after the 7 day coculture period. This revealed that a significantly higher proportion of cells generated in the micromarrows (2.64% ± 0.4%) possessed the LSK phenotype compared with 2D controls (1.68% ± 0.4%) ($p = 0.0021$) (Fig. 3C). Calculation of total numbers of LSK generated per culture revealed that approximately twice as many LSK were present in the 3D culture system compared with the 2D counterparts (3D micromarrows: $24.1\% \pm 5.7 \times 10^3$ vs. 2D controls: $12.2 \pm 2.3 \times 10^3$ LSK) ($p = 0.0006$). This subsequently translated into a significantly higher LSK fold expansion when LSK-MSC cocultures are performed in 3D (3D micromarrows: 4.0 ± 0.9-fold; 2D controls: 2.0 ± 0.5- fold) ($p = 0.0006$) (Fig. 3D).

Hematopoietic cells are randomly distributed in and around aggregates

The distribution of hematopoietic cells within the aggregates was examined by collecting the cultures without disturbing the aggregates and passing the suspension through a 30 μm cell strainer to remove cells no longer bound to the aggregates. Using this technique, it was found that only 3.8% ± 1.9% of the total collected cells were still bound within aggregates. Upon flow cytometric analysis of this fraction it was found that similar percentages of LSK were within the aggregates (3.8% ± 2.2%), meaning that cells with the LSK phenotype were evenly dispersed between the aggregates and unbound cells. The distribution of MSC and hematopoietic cells from the 7 day coculture product was further

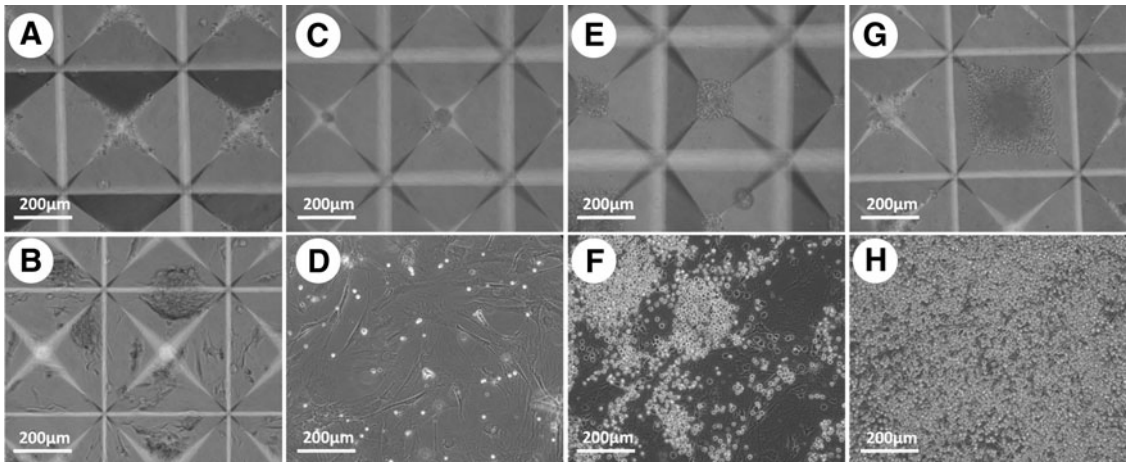

FIG. 2. Phase contrast microscopy of micromarrow and traditional 2D cultures over time. **(A)** Lineage^{dim}, Sca-1^{+} and c-kit^{+} (LSK)–mesenchymal stem/stromal cell (MSC) cocultures were initiated in 3D aggregates in discrete microwells using the AggreWell product. **(B)** It was found that the surface of this product did not prevent cell attachment and thus the surface was modified using a ML technique to prevent this occurrence. Three-dimensional micromarrow cocultures were visualized after **(C)** 1 day, **(E)** 5 days, and **(G)** 7 days of culture by light microscopy. Likewise, adherent 2D cocultures were initiated on monolayers of MSC and were observer after **(D)** 1 day, **(F)** 5 days, and **(H)** 7 days of culture by light microscopy to contrast between the different geometries. All images are by phase contrast microscopy and ×10 magnification.

confirmed by immunofluorescence assessment using GFP^{+} MSC (green) and the pan hematopoietic cell marker CD45 (red) (Fig. 4A–H). The cores of the aggregates were composed of MSC (GFP+) and this population was not observed outside the core (Fig. 4A, E). Consistent with the flow cytometry counting data, most of the hematopoietic cells (GFP- CD45+) were physically detached from the core (Fig. 4B, D). However, a significant population of hematopoietic

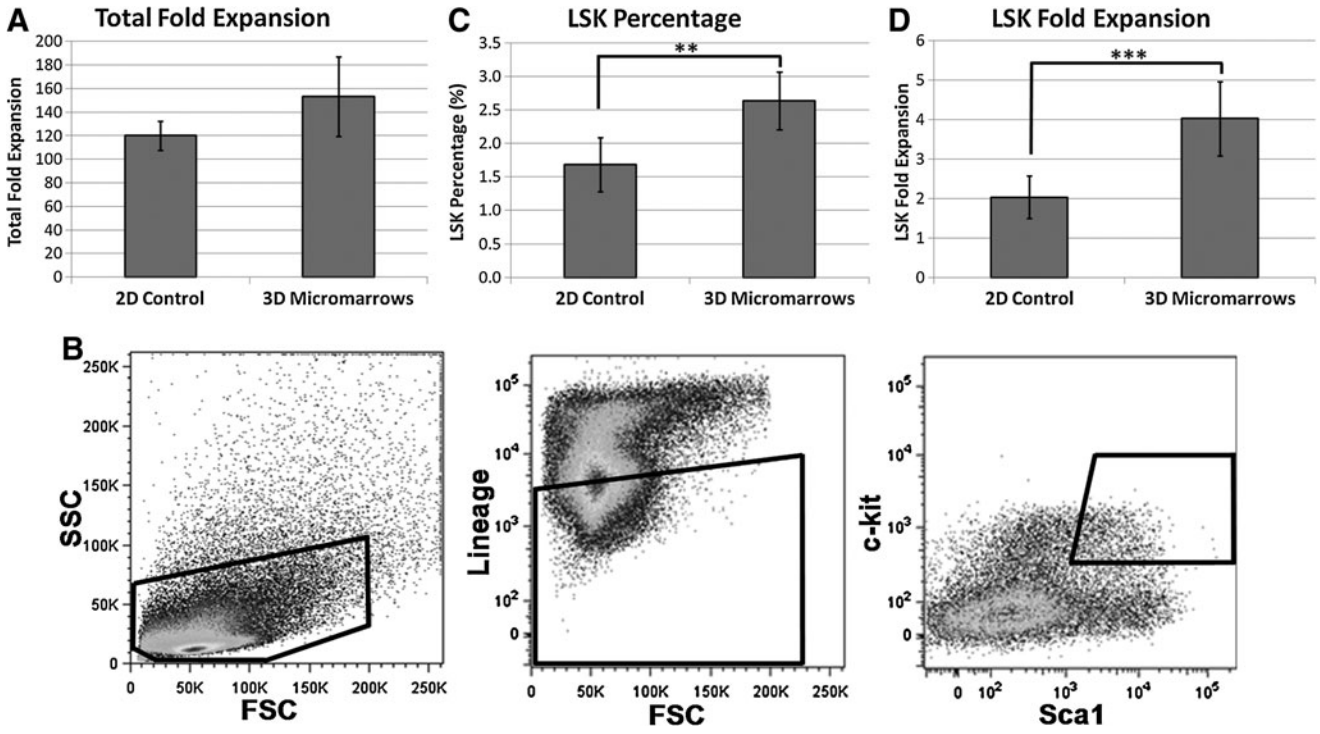

FIG. 3. Quantification of LSK-MSC cocultures in 3D aggregates or 2D adherent expansion cultures. **(A)** Total cell expansion after 7 days was equivalent in 3D aggregates and 2D adherent expansion cocultures ($n=7$). **(B)** The final cell culture product was assessed by flow cytometry for the LSK phenotype. **(C)** Quantification of the LSK phenotype revealed that a significantly higher percentage of LSK were present when the LSK–MSC cocultures were performed in a 3D aggregate geometry compared with the 2D adherent geometry ($p=0.0021$; $n=7$). **(D)** The product of total cell expansion and proportion of LSK in the cultures showed that expansion of LSK cells was significantly higher in the 3D aggregate cultures ($p=0.0006$; $n=7$). Graphical data is mean ± standard deviation.

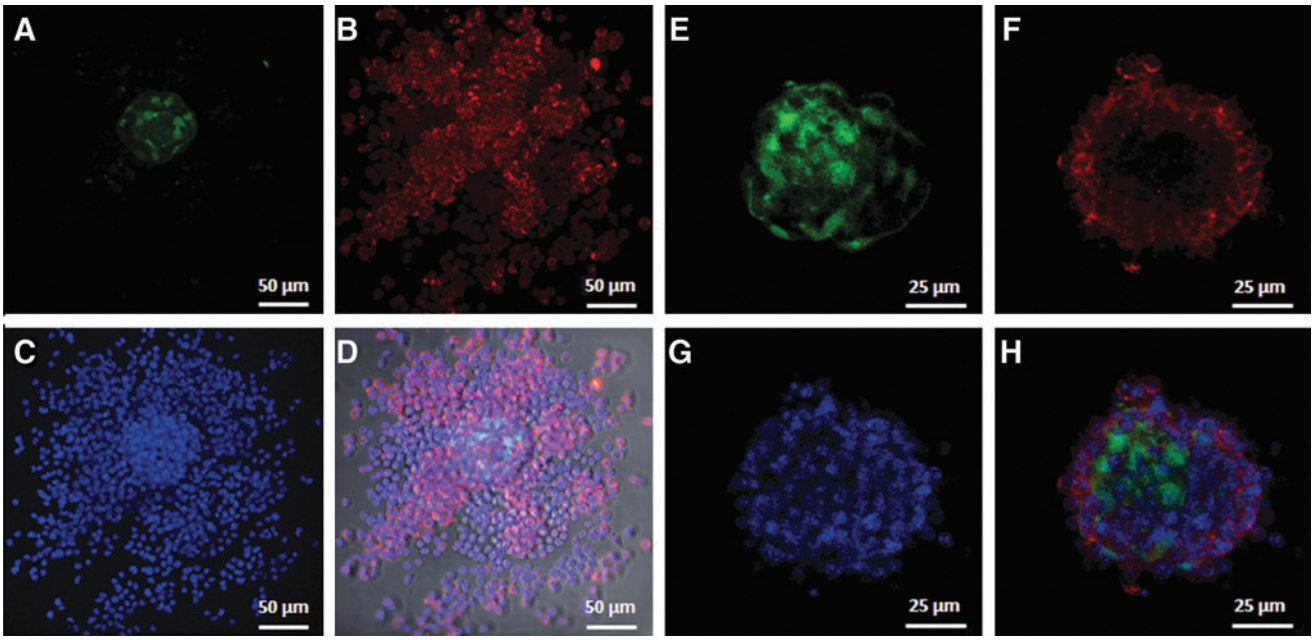

FIG. 4. Hematopoietic cell distribution within the micromarrows. The distribution of cells within or around the aggregates was determined using differential staining of green fluorescent protein (GFP+) MSC (green) and immunofluorescent labeling of CD45+ hematopoietic cells (red) derived from C57BL/6 mice. **(A–D)** This was first identified by assessing the gross morphology of the 7 day culture. **(E–H)** Conversely, images through the transverse plain of the central aggregates were used to more closely elucidate cell distribution within the aggregate itself. **(A, E)** This revealed that MSC (GFP+) remain bound to each other and form a central cell-derived scaffold in the cultures. MSC were not observed outside the core. Assessment of the distribution of CD45+ hematopoietic cells demonstrated that, **(B)** while most of these detached from the core structure, **(F)** a proportion remain bound and persisted primarily around the periphery of the MSC aggregate. **(C, G)** DAPI (blue) staining was used to identify the nuclei of all cells. **(D, H)** A merge of GFP+ MSC, CD45+ hematopoietic cells, and DAPI nuclei was used to determine each cell's relative location. Images ×20–25 optical magnification. DAPI, 4',6-diamidino-2-phenylindole.

cells remained bound to the MSC core although they were largely distributed toward the periphery of the aggregate (Fig. 4F, H). Intriguingly, we noted that there were some hematopoietic cells retained within the central region of the aggregates, where the relative MSC density was greater. Presumable the hematopoietic cells retained within the aggregate core would benefit from greater direct contact with the supportive MSC population.

MSC maintained in 3D express higher levels of HSC niche markers

In hopes of explaining the greater LSK expansion observed in the micromarrows, we performed qRT-PCR to assess the relative expression of the key HSC niche markers angiopoietin-1 (*Ang-1*), angiopoieitin-2 (*Ang-2*), stem cell factor (*SCF*), jagged-1, and stromal cell-derived factor-1 (*SDF-1*) which are known to be essential for HSC survival *in vivo* and to be expressed by niche support cells.[26] MSC were cultured alone either in 3D aggregates or in 2D monolayers using the same cell seeding density and medium as described for the previous experiments. These cells were harvested either after 4 or 7 days of culture and relative gene expression assessed. This revealed that MSC maintained in aggregates always expressed significantly higher levels of *Ang-1*, *SCF*, jagged-1, and *SDF-1* both after 4 and 7 days of culture ($p<0.05$) (Fig. 5A, C–E). Expression of *Ang-2* was also significantly higher in 3D cultures after 4 days of culture ($p<0.05$), but was equivalent to 2D cultures after 7 days (Fig. 5B).

Discussion

The central aim of this study was to develop a novel 3D HSC-MSC coculture platform that would enable better replication of the HSC niche and consequently enhance *in vitro* HSC culture outcomes. To do this, 3D aggregates of murine HSPC (LSK) and MSC, which we termed "micromarrows," were formed on a specially designed nonadherent microwell surface. It was hypothesized that establishing cocultures in cell only structures would enhance cell–cell interaction and eliminate the negative influence of plastic culture surfaces known to perturb cell function.[9]

Our cultures were established using the AggreWell product which was originally designed for the generation of embryoid bodies.[13] Using this surface allowed the generation of 1200 uniform aggregates in each well of the 24-well AggreWell plate. In contrast, existing methods to generate scaffold-free aggregates of cells, such as the hanging drop method, would require over twelve 96-well plates to replicate a single AggreWell culture. Thus, this method provides a high-throughput method for cellular aggregate formation. In using the AggreWell product we found that surface modification was necessary to prevent cell attachment and spreading. Core to our hypothesis is that cell-surface interactions need to be minimized and cell–cell interactions encouraged to generate a more niche like 3D environment. Using the surface modified products, micromarrows rapidly self-assembled into stable spheroids which remained intact over the culture duration. Importantly, the multicellular

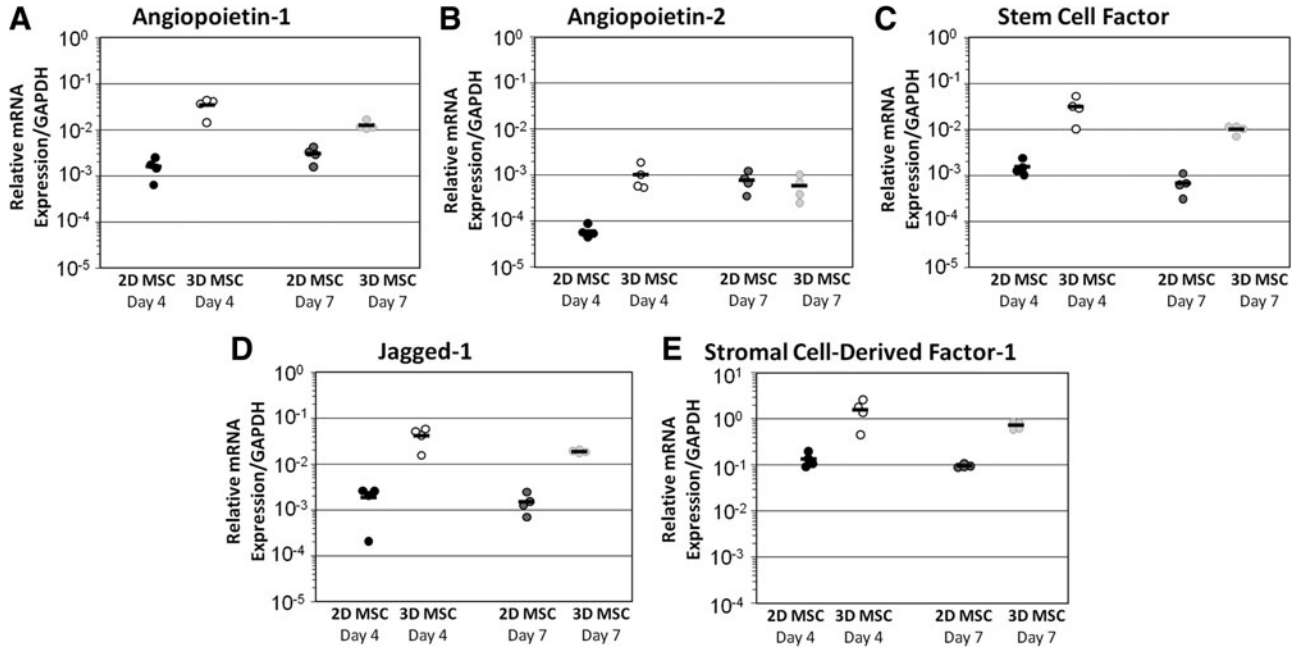

FIG. 5. Gene expression of hematopoietic stem cell (HSC) niche markers when MSC are maintained in 3D aggregates versus traditional 2D adherent cultures. MSC alone were grown in either the AggreWell system or as 2D monolayers for either 4 or 7 days at which point they were harvested and analyzed for expression of the key HSC niche markers **(A)** angiopoietin-1, **(B)** angiopoietin-2, **(C)** stem cell factor **(D)** jagged-1, and **(E)** stromal cell-derived factor-1. In all cases, except angiopoietin-2 at day 7, expression of these markers was significantly higher when MSC were cultured in 3D aggregates ($p < 0.05$; $n = 4$).

aggregates that were formed were uniform in size which demonstrated that cells were evenly seeded into each microwell using this method. Coculture of LSK in 3D aggregates did not affect the total hematopoietic cell proliferation relative to 2D controls. However, it was found that the proportion, and therefore the yield, of LSK within 3D cultures were significantly greater than that which could be achieved in 2D cocultures. This would suggest that the 3D micromarrow environment supports greater HSPC self-renewal. This result is not surprising given that qRT-PCR of the MSC populations cultured either in 2D or in 3D revealed that key HSC maintenance factors (*Ang-1*, *Ang-2*, *SCF*, Jagged, and *SDF-1*) are significantly upregulated when the cells are maintained in 3D aggregates. Micromarrows assembled within a few hours of being seeded and, through cell-sorting thermodynamics, most of the hematopoietic cells produced appeared to have detached from the central MSC aggregate and existed without direct cell-cell contact with MSC. Further, a ring of hematopoietic cells was found around the MSC core at day 7.

Interestingly we also observed that some hematopoietic cells, which we speculate represent a more primitive subpopulation, were maintained within the core of the aggregates over the 7 day culture period. In contrast, we hypothesize that maturing cells are pushed to the surface of the micromarrows and are ultimately ejected from the aggregates where they settle in the microwell adjacent to the aggregate. We hope to prove this in future work by studying the cell sorting thermodynamics of the MSC support cells when they are seeded with various subpopulations of purified hematopoietic cells. In such an experiment we might hypothesize that the organization of the 3D aggregate would reflect the engraftment potential of the selected hematopoietic population. Similarly, we can envision that this study could evolve to using purified populations of stromal support cells, individually or in combinations, to reconstruct the niche in microaggregates and investigate engraftment mechanisms. These concepts are not completely original as other groups have attempted to study HSC migration behavior *in vitro* using cell aggregates. Previously, de Barros *et al*. reported the formation of large human-derived MSC aggregates and the migration of patterns of $CD34^+$ HSPC populations throughout these preformed aggregates.[18] Similarly, Bug *et al.* presented a cross-species spheroid coculture assay for examination of specific signaling cascades involved in hematopoietic cell migration. In this system human cord blood $CD34^+$ cells were seeded with preformed aggregates of the murine stromal cell line M2-10B4.[18] Both of these studies utilized 96-well plate systems, modified to be nonadherent, to generate single macroaggregates having an approximate diameter of 400 μm. We believe our system, which produces ~1200 microaggregates per well (in a 24-well plate) where each aggregate has a diameter of ~50–100 μm, may accelerate such studies. In such an assay aggregate cocultures could be established using an adaptation of the strategy described by de Barros *et al.*[18] where MSC aggregate formation is followed by the subsequent seeding of HSPC or by using our method where MSC and HSPC are formed into an aggregate in a single step. In either case, the temporal distribution of the HSPC within the aggregate may provide an indication of the engraftment capacity of the specific hematopoietic subpopulation used in the aggregation assay.

Herein we have presented a system enabling the efficient establishment of an HSPC–MSC 3D coculture that is free of biomaterial interactions. This noncomplex culture system

allows for the simultaneous seeding of HSPC and MSC into the 3D aggregates that subsequently undergo a cell sorting thermodynamic process to achieve their natural spatial 3D organization. In this study we have utilized a hematopoietic growth factor-free media that enables us to purely exploit and evaluate the supportive capacity of the support stromal cells. Our controls show that no live hematopoietic cells could be observed after 7 days when the stromal cells were absent from the culture, thus indicating that hematopoietic cell growth and survival is dependent on the presence of the stromal cells in our system rather than factors provided by the FBS. In the absence of other factors, it is clear that the 3D organization offered by the micromarrows is superior to classic 2D HSPC-MSC coculture. This 3D scaffold-free culture of HSPC is a platform for niche dissemination and provides a model for homing/engraftment. Finally, as demonstrated in this paper, it is also a suitable platform for the expansion of hematopoietic cell populations. Thus, this system represents an important first step toward a high-throughput 3D coculture system that may enable HSC niche recapitulation and potentially extensive *in vitro* HSC self-renewal.

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Disclosure Statement

The authors declare no conflicts of interest.

References

1. Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature **466,** 829, 2010.
2. Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., and Bianco, P. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell **131,** 324, 2007.
3. Dexter, T.M., Allen, T.D., and Lajtha, L.G. Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. J Cell Physiol **91,** 335, 1977.
4. Mesoblast_Media_Release. Successful Bone Marrow Regeneration In Cancer Patients Using Cord Blood Expanded By Proprietary Stem Cells. Melbourne, Australia: Mesoblast Ltd., 2009.
5. Jaroscak, J., Goltry, K., Smith, A., Waters-Pick, B., Martin, P.L., Driscoll, T.A., Howrey, R., Chao, N., Douville, J., Burhop, S., Fu, P., and Kurtzberg, J. Augmentation of umbilical cord blood (UCB) transplantation with *ex vivo*-expanded UCB cells: results of a phase 1 trial using the AastromReplicell System. Blood **101,** 5061, 2003.
6. McNiece, I., Jones, R., Bearman, S.I., Cagnoni, P., Nieto, Y., Franklin, W., Ryder, J., Steele, A., Stoltz, J., Russell, P., McDermitt, J., Hogan, C., Murphy, J., and Shpall, E.J. *Ex vivo* expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer. Blood **96,** 3001, 2000.
7. Pecora, A.L., Stiff, P., LeMaistre, C.F., Bayer, R., Bachier, C., Goldberg, S.L., Parthasarathy, M., Jennis, A.A., Smith, A.K., Douville, J., Chen, B., Armstrong, R.D., Mandalam, R.K., and Preti, R. A phase II trial evaluating the safety and effectiveness of the AastromReplicell system for augmentation of low-dose blood stem cell transplantation. Bone Marrow Transplant **28,** 295, 2001.
8. Stiff, P., Chen, B., Franklin, W., Oldenberg, D., Hsi, E., Bayer, R., Shpall, E., Douville, J., Mandalam, R., Malhotra, D., Muller, T., Armstrong, R.D., and Smith, A. Autologous transplantation of *ex vivo* expanded bone marrow cells grown from small aliquots after high- dose chemotherapy for breast cancer. Blood **95,** 2169, 2000.
9. Jacks, T., and Weinberg, R.A. Taking the study of cancer cell survival to a new dimension. Cell **111,** 923, 2002.
10. Markway, B.D., Tan, G.K., Brooke, G., Hudson, J.E., Cooper-White, J.J., and Doran, M.R. Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. Cell Transplant **19,** 29, 2010.
11. Wang, W., Itaka, K., Ohba, S., Nishiyama, N., Chung, U.I., Yamasaki, Y., and Kataoka, K. 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. Biomaterials **30,** 2705, 2009.
12. Jahn, K., Richards, R.G., Archer, C.W., and Stoddart, M.J. Pellet culture model for human primary osteoblasts. Eur Cell Mater **20,** 149, 2010.
13. Ungrin, M.D., Joshi, C., Nica, A., Bauwens, C., and Zandstra, P.W. Reproducible, ultra high- throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. PLoS One **3,** e1565, 2008.
14. Sasaki, T., Takagi, M., Soma, T., and Yoshida, T. 3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics. Cytotherapy **4,** 285, 2002.
15. Takagi, M., Iemoto, N., and Yoshida, T. Effect of concentrations of murine stromal and hematopoietic cells on the progenitors expansion in their three-dimensional coculture in nonwoven fabrics. J Biosci Bioeng **94,** 365, 2002.
16. Zhang, Y., Chai, C., Jiang, X.S., Teoh, S.H., and Leong, K.W. Co-culture of umbilical cord blood $CD34^+$ cells with human mesenchymal stem cells. Tissue Eng **12,** 2161, 2006.
17. Riordan, S., and Williams, R. Bioartificial liver support: developments in hepatocyte culture and bioreactor design. Br Med Bull **53,** 730, 1997.
18. de Barros, A.P., Takiya, C.M., Garzoni, L.R., Leal-Ferreira, M.L., Dutra, H.S., Chiarini, L.B., Meirelles, M.N., Borojevic, R., and Rossi, M.I. Osteoblasts and bone marrow mesenchymal stromal cells control hematopoietic stem cell migration and proliferation in 3D *in vitro* model. PLoS One **5,** e9093, 2010.
19. Bug, G., Rossmanith, T., Henschler, R., Kunz-Schughart, L.A., Schroder, B., Kampfmann, M., Kreutz, M., Hoelzer, D., and Ottmann, O.G. Rho family small GTPases control migration of hematopoietic progenitor cells into multicellular spheroids of bone marrow stromal cells. J Leukoc Biol **72,** 837, 2002.
20. Timmins, N.E., and Nielsen, L.K. Generation of multicellular tumor spheroids by the hanging- drop method. Methods Mol Med **140,** 141, 2007.

21. Boxshall, K., Wu, M.H., Cui, Z., Cui, Z.F., Watts, J.F., and Baker, M.A. Simple surface treatments to modify protein adsorption and cell attachment properties within a poly (dimethylsiloxane) micro-bioreactor. Surface and Interface Analysis **38,** 198, 2006.
22. Croll, T.I., O'Connor, A.J., Stevens, G.W., and Cooper-White, J.J. A blank slate? Layer-by-layer deposition of hyaluronic acid and chitosan onto various surfaces. Biomacromolecules **7,** 1610, 2006.
23. Doran, M.R., Frith, J.E., Prowse, A.B., Fitzpatrick, J., Wolvetang, E.J., Munro, T.P., Gray, P.P., and Cooper-White, J.J. Defined high protein content surfaces for stem cell culture. Biomaterials **31,** 5137, 2010.
24. Wagers, A.J., Allsopp, R.C., and Weissman, I.L. Changes in integrin expression are associated with altered homing properties of Lin(-/lo)Thy1.1(lo)Sca-1(+)c-kit(+) hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. Exp Hematol **30,** 176, 2002.
25. Wilson, A., Oser, G.M., Jaworski, M., Blanco-Bose, W.E., Laurenti, E., Adolphe, C., Essers, M.A., Macdonald, H.R., and Trumpp, A. Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci **1106,** 64, 2007.
26. ter Huurne, M., Figdor, C.G., and Torensma, R. Hematopoietic stem cells are coordinated by the molecular cues of the endosteal niche. Stem Cells Dev **19,** 1131, 2010.

Address correspondence to:
Michael Doran, B.Sc., B.Eng., Ph.D.
Stem Cell Therapies Laboratory
Institute of Health and Biomedical Innovation
Queensland University of Technology
60 Musk Ave.
Kelvin Grove
Brisbane
Queensland 4059
Australia

E-mail: michael.doran@qut.edu.au

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