HHS Public Access

Author manuscript

Biomed Microdevices. Author manuscript; available in PMC 2019 June 26.

Published in final edited form as:

Biomed Microdevices. ; 20(1): 13. doi:10.1007/s10544-017-0255-3.

Stromalized microreactor supports murine hematopoietic progenitor enrichment

Danika Khong[1], **Matthew Li**[1], **Amy Singleton**[1], **Ling-Yee Chin**[1], and **Biju Parekkadan**[1,2,3]

[1]Department of Surgery, Center for Surgery, Innovation, & Bioengineering, Massachusetts General Hospital, Harvard Medical School and the Shriners Hospitals for Children, Boston, MA 02114, USA

[2]Department of Biomedical Engineering, Rutgers University and the Department of Medicine, Rutgers Biomedical and Health Sciences, Piscataway, NJ 08854, USA

[3]Harvard Stem Cell Institute, Cambridge, MA 02138, USA

Abstract

There is an emerging need to process, expand, and even genetically engineer hematopoietic stem and progenitor cells (HSPCs) prior to administration for blood reconstitution therapy. A closed-system and automated solution for ex vivo HSC processing can improve adoption and standardize processing techniques. Here, we report a recirculating flow bioreactor where HSCs are stabilized and enriched for short-term processing by indirect fibroblast feeder coculture. Mouse 3 T3 fibroblasts were seeded on the extraluminal membrane surface of a hollow fiber micro-bioreactor and were found to support HSPC cell number compared to unsupported BMCs. CFSE analysis indicates that 3 T3-support was essential for the enhanced intrinsic cell cycling of HSPCs. This enhanced support was specific to the HSPC population with little to no effect seen with the Lineage^{positive} and Lineage^{negative} cells. Together, these data suggest that stromal-seeded hollow fiber micro-reactors represent a platform to screening various conditions that support the expansion and bioprocessing of HSPCs ex vivo.

Keywords

Ex vivo expansion; Hematopoietic stem and progenitor cells; Bioprocessing

1 Introduction

Hematopoietic stem cells (HSCs) have long led the charge for using cells as therapies through their ability to reconstitute every blood cell in a the human body (Lorenz et al. 1951). Bone marrow, peripheral blood-mobilized, or umbilical cord HSCs are all viable cell sources that are harvested from patients and prepared for intravenous administration to restore hematopoiesis. The use of HSC transplants have been on the rise with new methods

Biju Parekkadan, biju_parekkadan@hms.harvard.edu.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10544-017-0255-3) contains supplementary material, which is available to authorized users.

of mobilization (Domingues et al. 2017; Liles et al. 2003), engraftment (Rak et al. 2017), ex vivo engineering (Zonari et al. 2017), and increasing ex vivo HSC expansion (Kiernan et al. 2017) all being collectively explored to expand the use of HSC therapy worldwide (Panch et al. 2017). Engineering technologies that can support each and all of these HSC bioprocessing steps can enable broad and standardized techniques that can scale to clinical centers both US and abroad for reproducible results.

The use of bioreactors for the ex vivo maintenance and/or engineering of HSCs is one potential technology to serve as a scalable platform. Various bioreactors formats exist and have been applied to HSC expansion with each having an issue that has potentially prevented wide scale use: continuous flow chambers allow for excellent nutrient delivery at the expense of increased, costly media consumption demand (Koller et al. 1993a; Koller et al. 1993b; Palsson et al. 1993; Sandstrom et al. 1996); stirred tanks support larger volumes and allow the monitoring of clonal growth and differentiation though have not maintained HSC phenotypes and stem potential (De Leon et al. 1998; Levee et al. 1994; Sardonini and Wu 1993; Zandstra et al. 1994); packed bed reactors allow for larger surface area to volume ratios enabling contact of cultured HSCs with growth ligands though are difficult to purify HSCs and have lower recovery yields (Liu et al. 2014; Meissner et al. 1999; Wang et al. 1995). Hollow fiber bioreactors have been used for continuous, recycling flow culture of HSCs though were not successful in supporting HSC numbers (Sardonini and Wu 1993; Schmelzer et al. 2015). While these systems have shown benefit, there still remains a need for an integrated system that maintains HSC numbers and phenotype with downstream ease of purification.

One major challenge in ex vivo bioprocessing of HSCs is the loss of a stem cell viability and/or phenotype over time in culture. Conventional culture methods rely heavily on media formulations to drive growth while maintaining the appropriate HSC phenotype. Currently, media supplements include various combinations of stem cell factor (SCF), interleukin- (IL) 3, –6, Fms-like tyrosine kinase-3 ligand (Flt3-L), granulocyte colony stimulating factor (G-CSF), fibroblast growth factor-132, Delta-1, and thrombopoietin which have been shown to expand mostly cord blood HSCs ex vivo (Bhatia et al. 1997; Conneally et al. 1997; Delaney etal. 2005; Himburg et al. 2010; Lui et al. 2014; Zhang et al. 2006). To enhance these ex vivo systems, the cellular HSC niche may be partially recapitulated through direct or indirect co-culture with fibroblastic stromal cells (Pan et al. 2017; Perucca et al. 2017) or endothelial cells (Gori et al. 2017). Advanced 3-D culture systems that more readily mimic the spatial organization of stromal and hematopoietic stem and progenitor cells (HSPCs) have shown enhanced long-term engraftment of expanded cells compared to 2-D cultures (Futrega et al. 2017).

This report describes a scalable hollow-fiber bioreactor system whereby a mouse embryonic fibroblast cell line, known to provide hematopoietic support (Roberts et al. 1987), was indirectly cocultured with mouse HSCs in a continuous, concentrated, and recycling flow to stabilize and enrich HSCs for short-term bioprocessing. This study builds on prior reports of direct coculture of HSCs with stromal cells whereby purification of HSCs is more difficult as cells were seeded on the extraluminal side of a hollow fiber membrane and subject to lower yields with additional downstream processing (Davis et al. 1996; Xue et al. 2014).

Since feeder cells were separated from HSCs by the hollow fiber membrane in our system, the isolation of HSCs was simplified. Proof-of-concept studies are presented to screen appropriate purified stromal feeder cells, develop a coculture method, and evaluation of the indirect stabilization and enrichment of mouse bone marrow HSCs.

2 Materials and methods

2.1 Animals and bone marrow cell suspension

Primary murine whole BM cells were isolated from 6 to 8 week old female C57 BL/6 mice femurs and tibia by flushing the central marrow using a 28-gauge needle and 3 mL syringe. Flushed marrow was triturated into a single cell suspension with a 22-gauge needle and 3 mL syringe and then subsequently filtered through a 70 μM nylon mesh to exclude tissue debris from the cell preparation. Cell were washed with culture media and then subjected to ammonium-chloride-potassium (ACK) lysis buffer (Biolegend, CA, USA) treatment for the removal of red blood cells. BM cells were then counted for experimental use using the cell viability dye, Trypan Blue (ThermoFisher Scientific, USA) and hemocytometer. Culture media was made with RPMI (Roswell Park Memorial Institute Medium; Gibco, USA) supplemented with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES (ThermoFisher Scientific, USA), and 1 mM Sodium Pyruvate (ThermoFisher Scientific, USA).

2.2 2-D Transwell co-culture

Initial optimization experiments were conducted in 2-D transwell co-cultures to allow a higher degree of throughput and parameter assessment to determine the optimal culture criteria: stromal cell type and dose. Corning 24-well plate transwells containing 0.4 μM pores (Grenier Biosciences, Austria) were used for these cultures. Stromal cells were seeded with either 2E5 (low stromal dose: 1:10) or 4E5 (high stromal cell dose: 1:2) cells, 24 h prior to whole bone marrow cell (BMC) addition. BMCs were seeded at either 2E6 or 8E5 cells respectively, and then the co-culture was incubated for 72 h at 37 °C. Cells were enumerated and analyzed by flow cytometry for their HSPC population. Cells were cultured with standard RPMI cell culture media as per description above.

2.3 3-D micro-reactor co-culture

2.3.1 Micro-reactor setup—The hollow fiber micro-reactor was purchased from Spectrum Labs (CA, USA). Sterilization began with gamma irradiation with 25–35 kGy at the manufacturing facility. The intra- and extra-capillary spaces of the micro-reactor hollow fibers were filled with sterile-filtered 0.5 M sodium hydroxide (NaOH) and left for 1 to 2 h at room temperature for reactor sterilization. The NaOH was flushed out multiple times with sterile phosphate buffered saline (PBS) (Sigma, USA). All fluidic connectors and tubing (Masterflex PharMed® BPT Tubing and Platinum-cured Silicone Tubing, IL, USA) were similarly soaked and flushed with 0.5 M NaOH for at least one hour, and washed with sterile-filtered PBS. Figure 2A illustrates the setup of the device for co-culture.

2.3.2 Micro-reactor seeding and sampling—Micro-reactors were seeded with either 7E6 (high stromal dose: 1:2) or 3 to 4E6 (low stromal dose: 1:10) 3 T3 stromal cells 24 h before co-culture was initiated. At 24 h, seeded micro-reactors were gently washed with

culture media, attached to cell culture bags (Origen) containing either 14E6 (high stromal dose: 1:2) or 30 to 40E6 (low stromal dose: 1:10) whole BMCs respectively. Flow was introduced by using a Masterflex (IL, USA) peristaltic pump which was run at 4 mL/min. 250–500 μL sample aliquots were taken at 1, 24, and 48 h timepoints for cell quantification and flow cytometric analyses. Co-cultures were harvested at 72 h after administering flow and analyzed similarly.

2.3.3 Stromal cell cultures—Murine mesenchymal stem cells were purchased from Gibco. Cells were subcultured in media composed of sterile α-MEM (Gibco, MA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Atlanta Biologicals, GA), 1% penicillin/streptomycin (Gibco, MA), 1% antibiotic-antimycotic (Gibco, MA) and used for experiments within 1–3 passages from the initial vendor stock. 3 T3 cells were purchased from the ATCC (American Type Culture Collection) and expanded as per their recommended instructions; in DMEM supplemented with 10% FBS (Atlanta Biologicals, GA) and 1% penicillin/streptomycin (Gibco, MA).

2.4 Flow cytometric analyses

Cells were stained for 20–30 min at 4 °C in the dark with directly labeled human monoclonal antibodies directed against CD11b-, CD11c-, Gr1-, CD3-, CD4-, CD8a-, CD19-, B220-, NK1.1, and TER119-conjugated to biotin, cKit (CD117)-APC, Sca1-PECy7, and streptavidin APC Cy7 (BD Biosciences and eBiosciences, USA). Cells were washed and then resuspended with PBS supplemented with 2% FBS and 2 mM Ethylenediaminetetraacetic acid (EDTA; Gibco, USA). Flow cytometric analysis was performed using FACS LSRII (BD Biosciences, USA) and FlowJo Software (USA).

Cell cycling was assessed with carboxyfluorescein (CFSE) incorporation at the start of the co-culture. F0 peaks were determined using the control sample of BMCs flowed through the micro-reactor without stromal support.

2.5 Statistical analyses

All statistical tests were performed using GraphPad Prism. Differences between groups were tested using Student's t-test whereby a p-value of ≤0.05 were considered to be statistically significant. All comparisons were performed relative to BM alone micro-reactor co-cultures. All experiments were repeated with at least three biological replicates.

3 Results

3.1 Murine embryonic fibroblast cell line supports LSK enrichment in 2-D non-contact dependent co-cultures

Stromal cells have previously been shown to enhance hematopoietic support of whole BMCs *in vitro* (Méndez-Ferrer et al. 2010; Morrison and Scadden 2014). We had two criteria for selecting an optimal stromal cell type: 1) their ease of cell isolation, maintenance, and expansion for "off-the-shelf" use as well as 2) their hematopoietic supporting potential of HSPCs. To determine which stromal cell type we would utilize for supporting whole BM cells *in vitro*, we compared HSPC supporting capacity of bone marrow mesenchymal

stromal cells (MSCs) to 3 T3 mouse embryonic skin fibroblast cell line. As a control, we included a non-stroma containing group. To screen for optimal co-culture conditions, we adapted a 2-D non-contact dependent transwell setup that mimics indirect coculture with higher throughput experimental advantage.

At 72 h after co-culture with and without stromal support, the whole BMC compartment was enumerated. Stromal support maintained the total number of whole BMCs (Fig.1A) at their initial seeding numbers of 2×10^6 input cells over 3 days. We then analyzed subpopulations of BMCs into the more therapeutically relevant LSK population, phenotypically defined as being $Lineage^{negative}$ $Sca^{positive}$ $cKit^{positive}$ (LSK) (Fig.1B). Interestingly, the 3 T3 fibroblast cell line exhibited a superior capacity to enrich for the LSK population, both within the $Lineage^{negative}$ and whole BMC pools (Fig.1B–C and Fig. S1). There were no phenotypic anomalies in the LSK profiles of 3 T3 stromal-supported BMCs (Fig.1C). These LSK enrichment findings were observed in a range of 1:2 and 1:10 of 3 T3 cells to whole BMCs (Fig. S1) suggesting a dynamic working range to scale up with. Our results indicate a previously undiscovered capacity for 3 T3 fibroblasts to support the enrichment of mouse HSPCs from whole BM. The “off-the-shelf” properties and superior-hematopoietic supporting potential led us to scale up with 3T3s for all subsequent experiments in our hollow fiber microreactor.

3.2 Hollow fiber microreactor allows scale up of mouse LSK expansion

Figure 2A–C outlines our hollow fiber bioreactor system. Bioreactors are traditionally employed for the scale-up of a single cell type. A hollow fiber system is used for streamlined exchange and replenishment of fresh media to support large scale cell expansion. This system was modified as a coculture tool wherein stromal cells are seeded in the extraluminal space while BMCs are flowed through the interior fiber lumen to investigate the potential of an engineered tissue layer to support LSKs during continuous suspension culture (Fig.2A–C).

The micro-reactor setup consists of cells flowing downward through specialized tubing from a gas-exchange bag through a Masterflex pump which drives the cells through hollow fibers via their intra-capillary inlet (Fig.2A–B). The extra-capillary surface was seeded with stromal cells through the marked inlets (Fig.2B). The hollow fiber membranes were composed of hydrophillic polyethersulfone (PES) and each fiber has a molecular weight cut off of 0.2 μM pores that allow the bidirectional exchange of only acellular fluid (Fig.2C). PES was selected for its highly durable nature in withstanding high temperatures and constant fluid exposure. Masterflex PharMed ® BPT Tubing was used to allow the flow of BMCs from the bag to the micro-reactor by looping through the Masterflex pump head (Fig. 2A). This PharMed BPT tubing is made from platinum silicone and was used because it demonstrates low levels of spallation, high resistance to acids and alkalis, and can withstand high pressures with minimal cell shearing. Flow rate was selected by testing the effect of 5 mL/min, 10 mL/min, and 15 mL/min on cell viability after 72 h of being subjected to flow (Fig.S2). Higher cell counts were observed at lower flow rates after the 72 h period, so the the lowest flow rate on the Masterflex pump of 4 mL/min was selected (Fig.S2).

To further investigate the effect of flow and BMC cell density during unsteady state conditions of reactor start up, we subjected whole BMCs to flow (4 mL/min) and analyzed the viability of cells 1 h later (Fig.2D–E). The total cell count after whole BMCs were subjected to 1 h of flow remained relatively unaltered, however there was a dramatic drop when the number of BMCs was increased to 30–40 × 10^6 cells (Fig.2D). The cell concentration remained the same in both seeding models: 2 × 10^6 cells/mL. Surprisingly, this drop was not accounted for by the detection of dead cells by exclusion dye, Trypan Blue (Fig.2E). In fact, a greater degree of cell death was detected in the lower cell-seeded device (Fig.2E). After the initial decline in counts seen with the higher cell-seeded model, the counts remained relatively constant throughout subsequent timepoints up to 72 h (Fig.S3). The lower cell volume-seeding model exhibited a progressive decline in cell number with increasing days under these conditions however this did not translate to any significant cell loss within the first 2 days in flow (Fig.S3). Prolonged exposure to flow and shear pressure did alter the frequency of cell death with both models, although also not statistically significant (Fig. S3; red bars). Since the most dramatic effect of flow and shear pressure on the seeded primary whole BMCs occurred within the first hour of being subjected to the peristaltic pump, all subsequent cell counts have been normalized to the 1 h timepoint to accurately reflect changes that were directly the result of the non-contact dependent stromal support and flow beyond the initial seeding.

3.3 Stromal cell support rescues cell loss in hollow fiber bioreactor

Static cocultures indicated that 3 T3 fibroblasts exhibited superior hematopoietic support over MSCs and un-supported BMCs (see Fig.1). To verify this result in our micro-reactor system, we seeded freshly-thawed 3T3s on the extraluminal surface of the hollow fibers 24 h prior to loading the device with primary BMCs to allow 3T3s time to attach and have stable function. 3 T3-containing co-cultures offered a statistically significant protective advantage over BMCs when compared to non-stromal supported marrow (Fig.3A). This was consistently seen until the 72 h timepoint. A high dose of stromal support was required of 1 3 T3 cell to 2 BMCs in order to maintain LSK numbers (Fig.3A–B). There were no observable differences in the numbers of dead cells detected over time suggesting that circulating cells likely adhered to system components and were unaccounted for in the suspension cell counts (Fig.S3–S4).

Total cell counts were included in the supplemental section. Similar to that seen with counts normalized to the 1 h samples, raw counts at the 1,24,48, and 72 h timepoints also indicated that 3 T3 fibroblasts at a dose of 1 stromal cell to 2 BMCs, were able to prevent the trending decline in cell number seen with non-supported BMCs (Fig.S3–4). Following this result, all subsequent experiments to dissect the hematopoietic compartment of the BMCs from the micro-reactor were conducted with the 1:2 high stromal dose model.

3.4 3 T3 fibroblasts enrich for LSKs

The ability of the 3 T3-seeded micro-reactor to stabilize cell counts for 48 h forms a strong foundation for its exploration to expand BM-derived cells; however a key therapeutic interest within this pool of cells is the hematopoietic stem and progenitor compartment. To evaluate this HSPC population, we analyzed the sampling timepoints by flow cytometry for

the LSK phenotype (Fig.4A–B). Our results indicate a very strong trend towards the enrichment of LSK cells within the whole BMC population from the 3 T3-seeded micro-reactors (Fig.4A). The LSK numbers were expanded in a similar fashion with 3 T3 support when compared to the non-stromal supported marrow (Fig.4B). A similar analysis was also performed with the low stromal dose device (Fig.S5). This LSK-enriching potential was only seen with the higher dose of 1 3 T3 cell to 2 BMCs (Fig.S5 and Fig.4A–B).

We next evaluated the stromal populations within the pool of whole BMCs: Lineage^{positive} and Lineage^{negative} cells. Interestingly, there were no detectable changes in the numbers of Lineage^{positive} and Lineage^{negative} hematopoietic-supporting cells within the whole BMC population (Fig.4C–D).

3.5 LSKs exhibit enhanced intrinsic cell cycling with 3 T3 support

Since we detected no differences within the stromal compartments of the whole BMCs that were used to flow through the 3 T3-seeded micro-reactors, we hypothesized that our observed enrichment of the LSK population was a result of intrinsic changes. We therefore pulsed the whole BMCs with CFSE prior to micro-reactor loading to investigate the intrinsic cell cycling patterns associated with 3 T3 support. Figure 5A illustrates the CFS^{lo} pool of LSKs analyzed. As expected, few LSKs were cycling at the 0 to 1 h timepoints (Fig.5B). At the later timepoints of 48 and 72 h, there were significantly (only 48 h timepoint was statistically significant) larger pools of LSKs that were CFSE^{lo}, when 3 T3 were supporting BMCs (Fig.5B).

To assess whether this was merely a result of all BM cells expanding within the 3 T3-seeded reactors, we further analyzed the cycling properties of the stromal Lineage^{positive} and Lineage^{negative} cells (Fig.5C–D). While there was a gradual increase of CFSE incorporation in both these cell types with increasing time in the micro-reactor, there were no differences between BMCs with and without stromal support (Fig.5C–D).

4 Discussion

The field of hematopoietic transplantation is now 60 years old, and we have found more applications for its therapeutic potential. Closed-system automated manufacturing challenges of HSCs are a new frontier to standardize the bioprocessing of HSCs for ex vivo expansion, manipulation and engineering. Bioengineered devices such as bioreactors offer potential solutions to this problem. The design of reactors that mimic *in vivo* conditions nurturing to HSC function; self-renewal for long-term hematopoietic, and multi-lineage reconstitution, and HSC expansion have so far proven promising.

In this study, whole BM cells from mice were used in a hollow fiber micro-reactor seeded with a mouse embryonic cell line, 3 T3, to assess the benefit, if any, such stromal support confers onto the HSPC population under conditions of flow. 3T3s have previously been shown to provide developmental support for fetal liver cells into mast cells (Irani et al. 1992). 3 T3-supported fetal liver cells exhibited improved survival, expansion, and differentiation (Irani et al. 1992; Levi-Schaffer et al. 1987). However few studies have investigated the HSPC-supporting capacity of 3T3s and in fact have reported conflicting

findings (Dormady et al. 2000; Roberts et al. 1987). We report for the first time, the ability of 3 T3 cells to support the enrichment of LSKs in a short-term culture within a hollow fiber micro-reactor. This methodology provides the field with a hollow fiber reactor scaled for proof-of-concept murine models allowing for the screening of conditions favorable for ex vivo cell manufacture.

While the concept of niche mimicry through co-culturing hematopoietic cells with their physiological stromal components is not novel, the application of flow to this system is (Gori et al. 2017; Pan et al. 2017; Perucca et al. 2017). In fact, physiological shear stress is believed to have anabolic impacts on the bone-lining osteoblasts and endothelial cells (Coughlin and Niebur 2012; Obi et al. 2014). Both these cell types lie in very close proximity to quiescent HSCs *in vivo* suggesting the potential involvement for flow on HSC development and function (Kumar and Geiger 2017). Only a handful of previous studies have investigated the effects of flow on hematopoietic-associated co-cultures within hollow fiber reactors (Davis et al. 1996; Xue et al. 2014). Both these studies showed success in expanding sub-purified Lineage^{negative} and purified CD34^{+} cells from human umbilical cord blood in hollow fiber bioreactors directly co-cultured with MSCs and endothelial cells respectively, and supplemented with cytokines. Xue et al. was able to demonstrate a 60-fold increase in CD34^{+} cell numbers and unaltered homing capacities through mice reconstitution studies (Xue et al. 2014).

These promising technologies are far from clinical practice however. Current standards for CD34^{+} expansion in clinical trials begin with the isolation of CD34^{+} from blood, umbilical cord blood, or BM. These cells are then subjected to pre-transplantation expansion ex vivo in at least 10-day long cultures that also rely heavily on the addition of expensive cytokines (Delaney et al. 2010; Shpall et al. 2002.; Wagner et al. 2016). While these systems require purification of CD34^{+} prior to expansion, our findings indicate that whole BMC co-cultures with stromal cells that mimic the in vivo crosstalk of secreted factors can lead to enrichment of the therapeutically relevant LSK population in both 2-D and 3-D models. In particular, we describe an LSK-enriching potential with the 3 T3 mouse embryonic fibroblast cell line. This observation suggests a culture system that does not require the cultivation of stromal cells that are challenging to isolate and manufacture themselves, thereby simplifying the requirements for the ex vivo expansion of LSKs (Davis et al. 1996; Xue et al. 2014). In trying to understand the nature of this enrichment, our enumeration of the supporting stromal populations; Lineage^{positive} and Lineage^{negative}, support the observation that 3 T3-support is specific to the LSK pool (Peris et al. 2015). In fact, only LSKs and neither Lineage expressing or non-expressing cells exhibited increased cycling properties with 3 T3-support.

With the excitement of targeted LSK enrichment, comes several technical hurdles that establishes the need to further optimize the hollow fiber micro-reactor setup for clinical use. One major hurdle that will need to be addressed is the loss of BMCs observed upon the initial application of flow. Despite this initial drop in cell counts, we observed that 3 T3 supported LSK numbers that exceeded both baseline-seeding levels as well as unsupported BMCs. Interestingly, we were unable to correlate this decline in cell numbers seen 1 h post-seeding to cell death, suggesting that even at the lowest flow rate of 4 mL/min there

potential cell shearing. In thinking of its clinical utility, this initial loss of cells will have to be addressed with either slower flow rates or larger tubing sizes to reduce shear pressure.

The increasing gap between supply and clinical demand of HSCs stipulates for the development of scalable bioengineering solutions that address 1) the expansion of relevant hematopoietic sub-populations, 2) the efficiency of culture times, and 3) the requirements for minimal cytokine supplementation. Our findings provide preliminary evidence for the ability for 3 T3-seeded hollow fiber micro-reactors to sustain whole BMC viability for up to 48 h while enriching for its LSK population providing a platform for HSC engineering and manufacturing under clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by the Shriners Hospitals for Children (B.P.), National Institutes of Health Grants R01EB012521 (B.P.). BP is a founder and equity holder of Sentien Biotechnologies, Inc. whom have licensed patents pertaining to MSC and extracorporeal device-based therapeutics. The authors declare no other competing financial interests.

References

Bhatia M, Bonnet D, Kapp U, Wang JCY, Murdoch B, Dick JE, Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J. Exp. Med 186, 619–624 (1997) [PubMed: 9254660]

Conneally E, Cashman J, Petzer A, Eaves C, Expansion *In Vitro* of Transplantable Human Cord Blood Stem Cells Demonstrated Using a Quantitative Assay of their Lympho-Myeloid Repopulating Activity in Nonobese Diabetic-Scid/Scid Mice. Proc. of the Natl. Acad. of Sci 94, 9836–9841 (1997)

Coughlin TR, Niebur GL, Fluid shear stress in trabecular bone marrow due to low-magnitude high-frequency vibration. J. Biomech 45, 2222–2229 (2012) [PubMed: 22784651]

Davis TA, Wiesmann W, Kidwell W, Cannon T, Kems L, Serke C, Delaplaine T, Pranger A, Lee KP, Effect of spaceflight on human stem cell hematopoiesis: Suppression of erythropoiesis and myelopoiesis. J. Leukoc. Biol 60, 69–76 (1996) [PubMed: 8699125]

De Leon A, Mayani H, Ramirez OT, Design, characterization and application of a minibioreactor for the culture of human hematopoietic cells under controlled conditions. Cytotechnology 28, 127–138 (1998) [PubMed: 19003414]

Delaney C, Varnum-Finney B, Aoyama K, Brashem-Stein C, Bernstein ID, Dose-dependent effects of the notch ligand Delta1 on ex vivo differentiation and *in vivo* marrow repopulating ability of cord blood cells. Blood 106, 2693–2699 (2005) [PubMed: 15976178]

Delaney C, Heimfeld S, Brashem-Stein C, Voorhies H, Manger RL, Bernstein ID, Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat. Med 16, 232–236 (2010) [PubMed: 20081862]

Domingues MJ, Nilsson SK, Cao B, New agents in HSC mobilization. Int. J. Hematol 105, 141–152 (2017) [PubMed: 27905003]

Dormady SP, Zhang X-M, Basch RS, Hematopoietic progenitor cells grow on 3T3 fibroblast monolayers that overexpress growth arrest-specific gene-6 (GAS6). Proc. Natl. Acad. Sci 97, 12260–12265 (2000) [PubMed: 11050245]

Futrega K, Atkinson K, Lott WB, Doran MR, Spheroid Coculture of hematopoietic stem/progenitor cells and monolayer expanded mesenchymal stem/stromal cells in polydimethylsiloxane

microwells modestly improves *in vitro* hematopoietic stem/progenitor cell expansion. Tissue Engineering Part C, Methods 23, 200–218 (2017) [PubMed: 28406754]

Gori JL, Butler JM, Kunar B, Poulos MG, Ginsberg M, Nolan DJ, Norgaard ZK, Adair JE, Rafii S, Kiem H-P, Endothelial cells promote expansion of long-term engrafting marrow hematopoietic stem and progenitor cells in primates. Stem Cells Transl. Med 6, 864–876 (2017) [PubMed: 28297579]

Himburg HA, Muramoto GG, Daher P, Meadows SK, Russell JL, Doan P, Chi JT, Salter AB, Lento WE, Reya T, et al., Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat. Med 16, 475–482 (2010) [PubMed: 20305662]

Irani AA, Craig SS, Nilsson G, Ishizaka T, Schwartz LB, Characterization of human mast cells developed *in vitro* from fetal liver cells cocultured with murine 3T3 fibroblasts. Immunology 77, 136–143 (1992) [PubMed: 1398760]

Kiernan J, Damien P, Monaghan M, Shorr R, McIntyre L, Fergusson D, Tinmouth A, Allan D, Clinical studies of ex vivo expansion to accelerate engraftment after umbilical cord blood transplantation: A systematic review. Transfus. Med. Rev 31, 173–182 (2017) [PubMed: 28087163]

Koller MR, Bender JG, Miller WM, Papoutsakis ET, Expansion of primitive human hematopoietic progenitors in a perfusion bioreactor system with IL-3, IL-6, and stem cell factor. Biotechnology (NY) 11, 358–363 (1993a)

Koller MR, Emerson SG, Palsson BO, Large-scale expansion of human stem and progenitor cells from bone marrow mononuclear cells in continuous perfusion cultures. Blood 82, 378–384 (1993b) [PubMed: 8329697]

Kumar S, Geiger H, HSC niche biology and HSC expansion ex vivo. Trends Mol. Med 23, 799–819 (2017) [PubMed: 28801069]

Levee MG, Lee GM, Paek SH, Palsson BO, Microencapsulated human bone marrow cultures: A potential culture system for the clonal outgrowth of hematopoietic progenitor cells. Biotechnol. Bioeng 43, 734–739 (1994) [PubMed: 18615796]

Levi-Schaffer F, Austen KF, Caulfield JP, Hein A, Gravallese PM, Stevens RL, Co-culture of human lung-derived mast cells with mouse 3T3 fibroblasts: Morphology and IgE-mediated release of histamine, prostaglandin D2, and leukotrienes. J. Immunol (Baltimore, md: 1950) 139, 494–500 (1987) [PubMed: 2439587]

Liles WC, Broxmeyer HE, Rodger E, Wood B, Hübel K, Cooper S, Hangoc G, Bridger GJ, Henson GW, Calandra G, et al., Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. Blood 102, 2728 (2003) [PubMed: 12855591]

Liu N, Zang R, Yang S-T, Li Y, Stem cell engineering in bioreactors for large-scale bioprocessing. Engineering in Life Sciences 14, 4–15 (2014)

Lorenz E, Uphoff D, Reid TR, Shelton E, Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Natl. Cancer Inst 12, 197–201 (1951) [PubMed: 14874130]

Lui WC, Chan YF, Chan LC, Ng RK, Cytokine combinations on the potential for ex vivo expansion of murine hematopoietic stem cells. Cytokine 68, 127–132 (2014) [PubMed: 24825677]

Meissner P, Schroder B, Herfurth C, Biselli M, Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells. Cytotechnology 30, 227–234 (1999) [PubMed: 19003372]

Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, MacArthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS, Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829–834 (2010) [PubMed: 20703299]

Morrison SJ, Scadden DT, The bone marrow niche for haematopoietic stem cells. Nature 505, 327–334 (2014) [PubMed: 24429631]

Obi S, Yamamoto K, Ando J, Effects of shear stress on endothelial progenitor cells. J. Biomed. Nanotechnol 10, 2586–2597 (2014) [PubMed: 25992410]

Palsson BO, Paek SH, Schwartz RM, Palsson M, Lee GM, Silver S, Emerson SG, Expansion of human bone marrow progenitor cells in a high cell density continuous perfusion system. Biotechnology (NY) 11, 368–372 (1993)

Pan X, Sun Q, Zhang Y, Cai H, Gao Y, Shen Y, Zhang W, Biomimetic macroporous PCL scaffolds for ex vivo expansion of cord blood-derived CD34+ cells with feeder cells support. Macromol. Biosci 17, 1700054 (2017)

Panch SR, Szymanski J, Savani BN, Stroncek DF, Sources of hematopoietic stem and progenitor cells and methods to optimize yields for clinical cell therapy. Biol. of Blood and Marrow Transplant 23, 1241–1249 (2017) [PubMed: 28495640]

Peris P, Roforth MM, Nicks KM, Fraser D, Fujita K, Jilka RL, Khosla S, McGregor U, Ability of circulating human hematopoietic lineage negative cells to support hematopoiesis. J. Cell. Biochem 116, 58–66 (2015) [PubMed: 25145595]

Perucca S, Di Palma A, Piccaluga PP, Gemelli C, Zoratti E, Bassi G, Giacopuzzi E, Lojacono A, Borsani G, Tagliafico E, et al., Mesenchymal stromal cells (MSCs) induce ex vivo proliferation and erythroid commitment of cord blood haematopoietic stem cells (CB-CD34+ cells). PLoS One 12, e0172430 (2017) [PubMed: 28231331]

Rak J, Foster K, Potrzebowska K, Talkhoncheh MS, Miharada N, Komorowska K, Torngren T, Kvist A, Borg A, Svensson L, et al., Cytohesin 1 regulates homing and engraftment of human hematopoietic stem and progenitor cells. Blood 129, 950–958 (2017) [PubMed: 27899358]

Roberts RA, Spooncer E, Parkinson EK, Lord BI, Allen TD, Dexter TM, Metabolically inactive 3T3 cells can substitute for marrow stromal cells to promote the proliferation and development of multipotent haemopoietic stem cells. J. Cell. Physiol. 132, 203–214 (1987) [PubMed: 3497927]

Sandstrom CE, Bender JG, Miller WM, Papoutsakis ET, Development of novel perfusion chamber to retain nonadherent cells and its use for comparison of human “mobilized” peripheral blood mononuclear cell cultures with and without irradiated bone marrow stroma. Biotechnol. Bioeng 50, 493–504 (1996) [PubMed: 18627011]

Sardonini CA, Wu Y-J, Expansion and differentiation of human hematopoietic cells from static cultures through small-scale bioreactors. Biotechnol. Prog 9, 131–137 (1993) [PubMed: 7763589]

Schmelzer E, Finoli A, Nettleship I, Gerlach JC, Long-term three-dimensional perfusion culture of human adult bone marrow mononuclear cells in bioreactors. Biotechnol. Bioeng 112, 801–810 (2015) [PubMed: 25335987]

Shpall EJ, Quinones R, Giller R, Zeng C, Baron AE, Jones RB, Bearman SI, Nieto Y, Freed B, Madinger N, et al., Transplantation of ex vivo expanded cord blood. Biol. of Blood and Marrow Transplant. 8, 368–376 (2002) [PubMed: 12171483]

Wagner JE, Brunstein CG, Boitano AE, DeFor TE, McKenna D, Sumstad D, Blazar BR, Tolar J, Le C, Jones J, et al., Phase I/II trial of StemRegenin-1 expanded umbilical cord blood hematopoietic stem cells supports testing as a stand-alone graft. Cell Stem Cell 18, 144–155 (2016) [PubMed: 26669897]

Wang TY, Brennan JK, Wu JH, Multilineal hematopoiesis in a three-dimensional murine long-term bone marrow culture. Exp. Hematol 23, 26–32 (1995) [PubMed: 7995368]

Xue C, Kwek KYC, Chan JKY, Chen Q, Lim M, The hollow fiber bioreactor as a stroma-supported, serum-free ex vivo expansion platform for human umbilical cord blood cells. Biotechnol. J 9, 980–989 (2014) [PubMed: 24449538]

Zandstra PW, Eaves CJ, Piret JM, Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells. Biotechnology (NY) 12, 909–914 (1994)

Zhang CC, Kaba M, Ge G, Xie K, Tong W, Hug C, Lodish HF, Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells. Nat. Med 12, 240–245 (2006) [PubMed: 16429146]

Zonari E, Desantis G, Petrillo C, Boccalatte FE, Lidonnici MR, Kajaste-Rudnitski A, Aiuti A, Ferrari G, Naldini L, Gentner B, Efficient ex vivo engineering and expansion of highly purified human hematopoietic stem and progenitor cell populations for gene therapy. Stem Cell Reports 8, 977–990 (2017) [PubMed: 28330619]

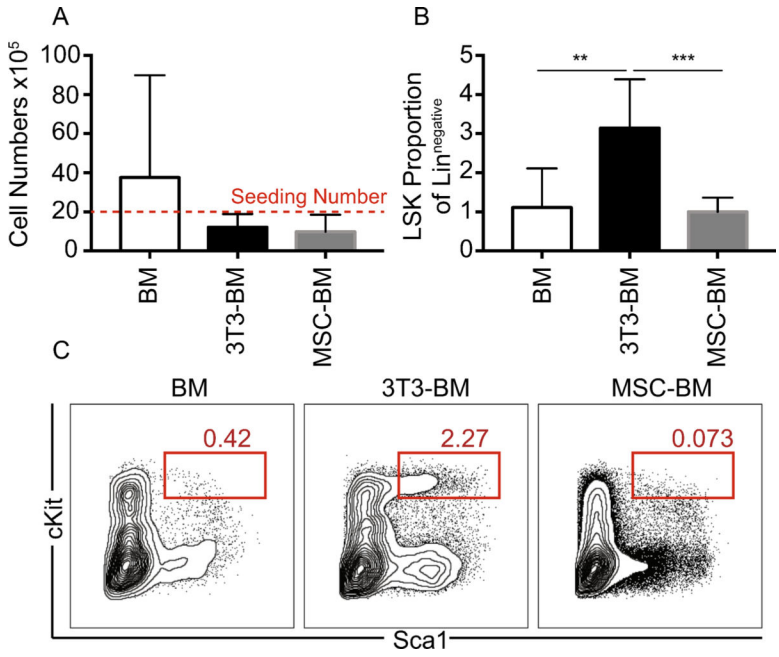

Fig. 1. 3 T3-supported 2-D short-term co-culture enriches for LSKs.

Analysis was performed on whole BMCs at a ratio of 1 stromal cell: 10 BMCs. Cells were cultured in a noncontact dependent manner for 72 h. **a**) Cell yields at the end of the 72 h culture period exhibit lower numbers with stromal-support. (**b**) LSK proportions of Lineage^{negative} show enrichment with 3 T3-support. (**c**) Representative gates of LSK cells from Lineage^{negative} population. All values are means + standard deviation. Data is representative of three biological replicates. * indicates p-values compared to BM alone. *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$

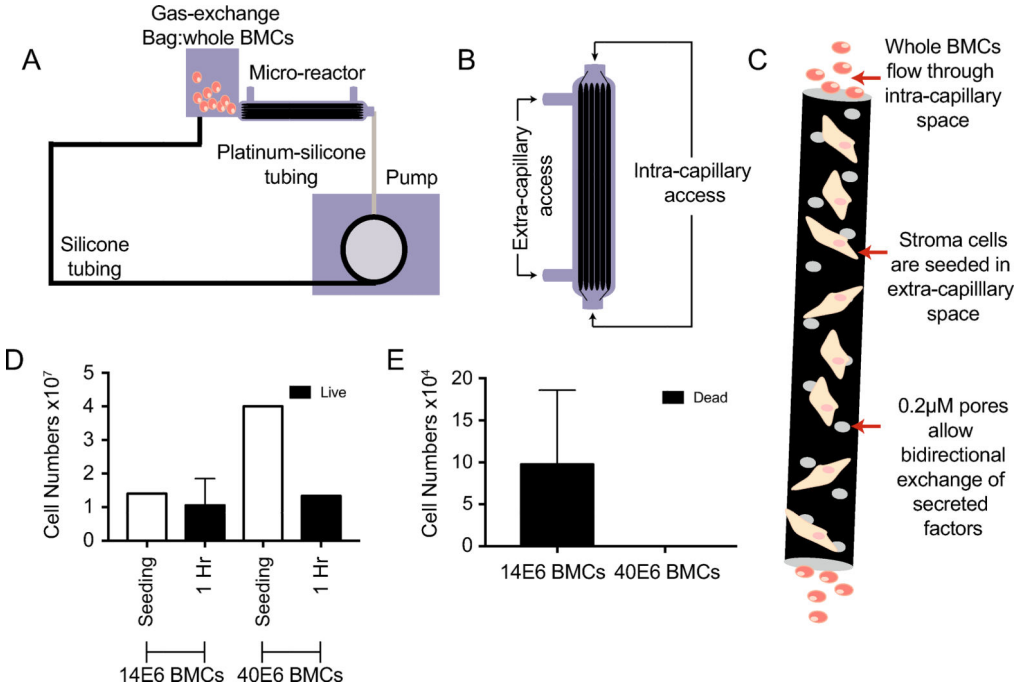

Fig. 2. Low cell volume-seeded hollow fiber micro-reactors show less cell loss.

Analysis was performed on whole BMCs after 1 h of device seeding. (**a**) Gas-exhachange cell bags containing whole BMCs were connected to intra-capillary space of the hollow fibers, which in turn was put through the Masterflex pump via platinum silicone pressure-resistant tubing and connected to the gas exchange bag. (**b**) Stromal cells were seeded into the extra-capillary space via the extra-capillary inlets. Whole BMCs were flowed through the inra-capillary space via the intra-capillary inlets. (**c**) There was no direct contact between stromal cells and whole BMCs. 0.2 μM pores along the hollow fiber surface allows the bi-directional exchange of secreted factors without allowing cells through. (**d**) Cell counts drop after 1 h of flow within the micro-reactor when 40E6 cells were seeded. No significant cell loss with the 14E6-seeded device. (**e**) More dead cells were detected with fewer cells seeded. All values are means + standard deviation. Data is representative of three biological replicates. * indicates p-values compared to BM alone. *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$

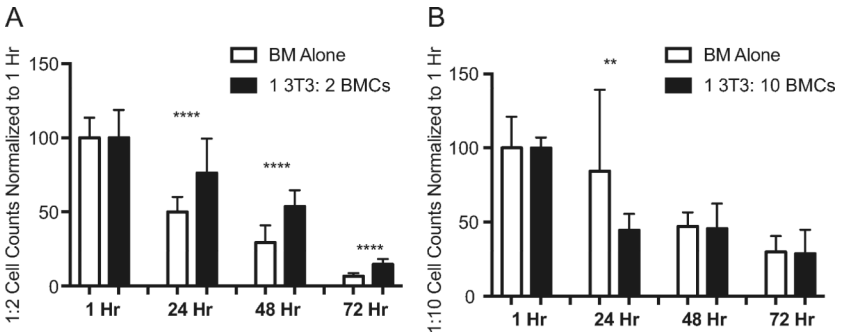

Fig. 3. High dose 3 T3-support recovers cell loss in microreactor.

Analysis was performed on whole BMCs at various timepoints after device seeding. Cell counts were normalized to the 1 h cell count. (**a**) High dose 3 T3 was able to significantly enhance cell numbers over unsupported BMCs. (**b**) Low dose 3 T3 was not able to rescue cell loss throughout all timepoints All values are means + standard deviation. Data is representative of three biological replicates. * indicates p-values compared to BM alone. *p <0.05, **p <0.01, and ***$p \leq 0.0001$.

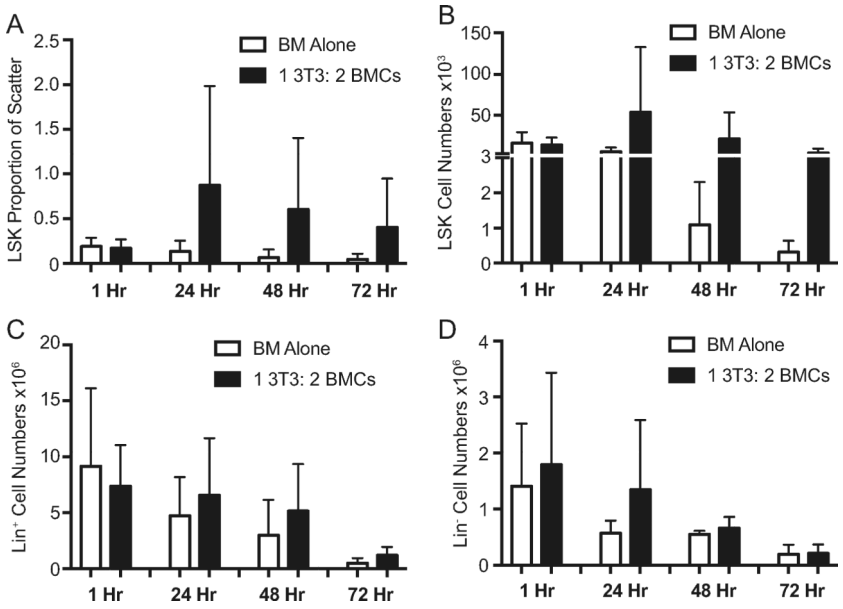

Fig. 4. 3 T3-mediated enrichment is specific to LSK population.

Analysis was performed on whole BMCs at various timepoints after device seeding. (**a**) LSK pool as a proportion of all live BMCs was enriched with 3 T3 support at all timepoints after 1 Hr. (**b**) LSK numbers were similarly higher with 3 T3 support at all timepoints after 1 Hr. (**c**) Lineage^{positive} population was unaltered with 3 T3 support and declined at 72 Hr. (**d**) Lineage^{negative} population was also unaltered with 3 T3 support and progressively declined with increasing time. All values are means + standard deviation. Data is representative of three biological replicates. * indicates p-values compared to BM alone. *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$

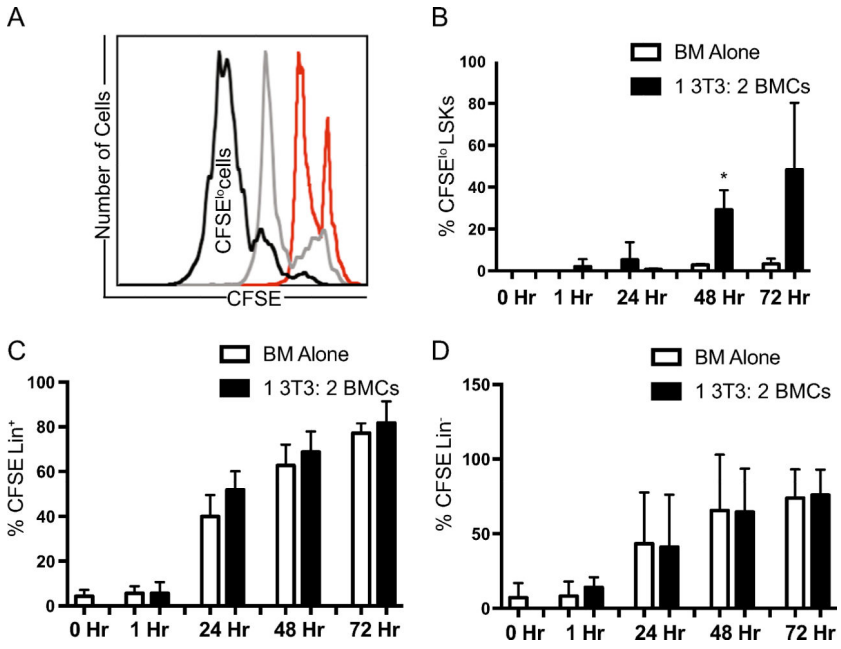

Fig. 5. 3 T3-mediated enhanced cell cycling was specific to LSK pool.

Analysis was performed on whole BMCs pulsed with CFSE. Cells were sampled at various timepoints for cell cycling properties. (**a**) Representative gating strategy for LSKs that were within $CFSE^{lo}$ population. (**b**) Proportion of LSKs that were $CFSE^{lo}$ was higher with 3 T3 support and increasing time in micro-reactor. (**c**) Proportion of $Lineage^{positive}$ that were CFSE+ increased with later timepoints. (**d**) Proportion of $Lineage^{negative}$ that were CFSE+ increased until 48 Hr and then stabilized. All values are means + standard deviation. Data is representative of three biological replicates. * indicates p-values compared to BM alone. *$p < 0.05$, **$p < 0.01$, and ***$p \leq 0.0001$