A bone marrow-on-a-chip that maintains hematopoietic regenerative capacity in vitro

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Dissertation

A BONE MARROW-ON-A-CHIP THAT MAINTAINS HEMATOPOIETIC
REGENERATIVE CAPACITY IN VITRO

by

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DEDICATION

This work is dedicated to the late Tara Gianoulis whose life was so unfairly cut short by a devastating illness. Her strength, compassion and brilliance will never cease being an inspiration to me and a constant reminder that the work of a physician-scientist is never done.
ACKNOWLEDGEMENTS

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A BONE MARROW-ON-A-CHIP THAT MAINTAINS HEMATOPOIETIC REGENERATIVE CAPACITY IN VITRO

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ABSTRACT

The bone marrow niche is composed of a complex set of cellular, chemical, structural and physical cues that are required to maintain viability and function of the hematopoietic system. [1-5]. The source of all differentiated blood cells, the hematopoietic stem cell (HSC), is housed within the protective confines of the bone marrow where the complex microenvironment regulates its ability to undergo self-renewal or to differentiate into all of the mature functional blood cell types that constitute the hematopoietic system [4-7]. Engineering an artificial bone marrow that reconstitutes the critical inductive cues of naturally occurring bone marrow in vivo that maintains them in vitro could lead to new models of hematopoietic diseases, as well as enable expansion of bone marrow for therapeutic transplantation and manufacturing of differentiated blood cell replacements. It has proven difficult, however, to identify or combine the correct set of biomaterials and biological signals necessary to recreate the complex bone marrow microenvironment or to maintain functional, multi-potent, self-renewing...
HSCs in culture [8-13]. Here, we describe a microfluidic bone marrow-on-a-chip created *in vivo* by combining microsystems and tissue engineering strategies to produce bone that contains a complex bone marrow niche. The hematopoietic compartment of the engineered bone marrow (eBM) has a distribution of HSCs, hematopoietic progenitor cells, and differentiated blood cell types that is virtually identical to natural marrow. Moreover, these hematopoietic populations are retained in normal proportions and the HSCs maintain their full regenerative capacity when the eBM is explanted and cultured in the microfluidic bone marrow chip *in vitro*. After four days of culture on-chip, hematopoietic cells isolated from the eBM engrafted a lethally-irradiated mouse, reconstituted the compromised bone marrow, and fully restored all differentiated blood cell lineages. Preliminary work with human umbilical cord blood (hCB) suggests that the bone marrow-on-a-chip platform may be extended beyond the mouse to support human HSCs and hematopoietic progenitors *in vitro*. This ability to engineer a complex bone marrow niche that is capable of maintaining functional HSCs offers new tools for expansion of cells for transplantation, manufacturing differentiated blood cells, evaluation of drug efficacy and toxicities, and study of hematopoietic diseases.
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<td>GFP</td>
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<td>HSC</td>
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INTRODUCTION

Hematopoietic stem cells (HSCs) are multi-potent cells defined by their ability to undergo self-renewal, remain undifferentiated or to develop into all of the mature functional blood cell types that constitute the hematopoietic system [1-4]. In an adult, bone marrow serves as the primary reservoir of HSCs and hematopoietic progenitor cells. Maintenance of an HSC in its delicate stem-like state requires a complex set of cellular, chemical, structural and physical cues found almost exclusively in the complex bone marrow microenvironment or niche (Figure 1) [1, 2, 5-7].

Figure 1 – Bone Marrow Microenvironment. Depiction of the complex physical, chemical, cellular and architectural features influencing HSCs and hematopoiesis in bone marrow microenvironment. Adapted from Wang et al.[2]
Given the critical importance of bone marrow in preserving and promoting hematopoietic function, engineering an artificial bone marrow that reconstitutes the essential inductive cues of naturally occurring bone marrow in vivo, that can be maintained in vitro would be of great value. An engineered bone marrow could serve as a platform to develop in vitro models of hematopoietic diseases, as well as enable expansion of blood components for therapeutic transplantation or test the efficacy and safety of drugs on individual patent marrow aspirates or peripheral blood samples. It has proven difficult, however, to identify or combine the correct set of biomaterials and biological signals necessary to recreate the complex bone marrow microenvironment or to maintain functional, multi-potent, self-renewing HSCs in culture [8-13].

**Bone Tissue Engineering**

*In vivo* ectopic bone formation has long been an active area of research. Previous work has demonstrated that implantation of demineralized bone into live animals results in the formation of new bone. It has been shown that new bone created from demineralized bone substrates occur through a step-wise process consisting of cellular recruitment, connective tissue deposition, vascularization, followed by the induction of multipotent, undifferentiated mesenchymal cells [14] to form chondroprogenitors, under low oxygen tension, and osteoprogenitors, under high oxygen tension [15-17]. Provided the appropriate environmental cues
or milieu, chondroprogenitors undergo further differentiation into chondrocytes, serving as cartilage producers, while osteoprogenitors differentiate into osteoblasts, initiating the process of mineralization and bone formation. Ultimately, the hematopoietic (monocyte)-derived osteoclasts arrive at the site of bone deposition to facilitate bone resorption and remodeling.

Investigators utilizing demineralized bone powder (DBP) to study ectopic bone formation *in vivo* often employ the strategy of introducing the bone-inducing materials intramuscularly or surgically into small bowel mesentery [16, 18-20]. While these strategies have resulted in bone and bone marrow formation, the limited marrow that forms within the tissue is heavily infiltrated with adipocytes, a cell type shown to have inhibitory effects on hematopoiesis [21], while displaying correspondingly low density of blood cells. Moreover, implantation of the bone-inducing materials inside of tissues (muscle, mesenchyme, etc.) results in bone of ill defined shape that is fully integrated into the tissue, rendering a structure that is not easily accessible or extractable from the animal.

DBP initiates bone formation largely through its *osteochonductive* properties, supplying a scaffold or surface on which new bone growth can be initiated. Depending on the preparation method of DBP, it can also be modestly *osteoinductive*, a property defined by the initiation of *de novo* bone formation through the recruitment and differentiation undifferentiated mesenchymal cells to become osteoprogenitor cells and ultimately bone producing osteoblasts [16].
The prototypical osteoinductive substrates include the family of bone morphogenic proteins (BMPs), a class of proteins originally discovered in bone powder [22]. While structurally similar, the individual isoforms are expressed during different stages of bone formation, suggesting differences in activity and function over the course of osteogenesis. Of the 30 BMPs identified, 5 have known osteoinductive properties (BMP-2, -4, -6, -7, and -9), stimulating endochondrial and intermembranous ossification [23]. BMP-2, for example, has been shown to be a potent stimulator of mesenchymal cell differentiation into, and maturation of, osteoblasts, thus facilitating the maintenance of normal bone mass [24]. Whereas, the expression of BMP-4 is reduced when cartilage resorption predominates and expressed at high levels during active osteogenesis [25]. Given the clinical interest in osteoinductive and osteoconductive materials for bone grafts and other orthopedic applications, there exist FDA-approved biomaterials consisting of bone matrix, natural, recombinant and synthetic structural proteins, and in some cases, BMPs to support ectopic bone formation in patients [20].

Organization of the Hematopoietic System

The hematopoietic system is organized in a hierarchical structure, whereby the most primitive of all blood cells, the HSC, serves as the cellular
precursor to all functional, differentiated blood cell types (Figure 2). The process of step-wise differentiation that occurs throughout hematopoiesis creates functional branch points in the hierarchy, signifying a restriction in developmental potential. A differentiating hematopoietic cell will pass through a series of potential-restricting branch points until a state of complete or terminal differentiation is achieved, producing mature, lineage-committed functional blood cell.

Under normal circumstances, the hematopoietic system is responsible for producing approximately one trillion blood cells per day in response to the needs of the body [26]. The ability to produce staggering quantities of many different blood cells types in a continuous and coordinated fashion is facilitated by this hierarchical structure. The step-wise differentiation behaves much like a signal transduction that employs a cascade of events to amplify the downstream effect. This results in an agile system characterized by rapid actuation and the potential for large-scale production of a given cell type. Furthermore, this structure offers the inherent benefit of requiring only a limited pool of self-renewing HSCs, while the more restricted progenitor cells with greater proliferative potential drive the production of differentiated cells, achieving optimal potential for self-renewal and differentiation.
Figure 2 – Hematopoiesis. Depiction of the hierarchical structure of hematopoiesis. All blood cells originate from a multipotent, self-renewing HSC and undergo a process of step-wise commitment, restricting the cell's potential while moving towards a terminally differentiated state (on right).

Hematopoietic Stem Cells and the Bone Marrow Niche

Postulated first in 1978, Schofield hypothesized that in order for a stem cell to remain undifferentiated, multipotent and capable of self-renewal, the cell must remain in a defined environment, or niche, that facilitates interactions with other cells and biological entities to regulate its fate, preventing it from differentiating and losing its "immortality" [27]. Since its initial proposal, Schofield’s stem cell niche hypothesis has largely been upheld.

In the adult, a large majority of the body's limited pool of HSCs resides and even seeks out, or 'homes' to, the bone marrow. There has been a great effort to define and characterize the anatomically defined, specialized regions or...
niches within the bone marrow that selectively support and regulate defined blood cell populations, especially the HSC and hematopoietic progenitors [28-32]. The two most widely studied anatomical sites of the bone marrow shown to harbor and maintain HSCs include the endosteal and perivascular niches [33].

**Endosteal Niche**

The endosteal niche, defined as the interface between the osteoblast-lined surface of the bone and the inner marrow cavity, is a site within the bone marrow where transplanted HSCs have been shown to home to or localize [34], and is critical to the retention of HSCs through ligand-receptor interactions including NCadherin [35] CXCL12 (stem cell derived factor-1, SDF-1) - CXCR4 [36, 37], and Ang1 - Tie2 [38]. Through a multitude of mechanisms including secretion of cytokines like granulocyte-colony stimulating factor (G-CSF) [39], expression HSC-supporting genes and signaling pathways including Wnt [40, 41] and Notch [42, 43], among many others [44], local regulation of calcium [45], and even response to input from the sympathetic nervous system [37], osteoblasts have been shown to function as positive regulators of HSCs [46].

To illustrate the integral role of the osteoblast in the HSC-supporting endosteal niche, transgenic mice that were designed to have increased numbers of osteoblast cells [35] or increased trabecular bone content [47] showed a concomitant increase in HSCs in the bone marrow. Selective depletion of
osteoblasts, on the other hand, resulted in impaired bone marrow or 'medullary' hematopoiesis, shifting the localization of HSCs and the burden of hematopoiesis to extramedullary sites (liver and spleen) to compensate for the apparent disruption in the endosteal niche [48].

The importance of osteoblasts and the endosteal niche to hematopoietic regulation is, perhaps, not surprising when considering the coordinated development of bone and blood illustrated by mammalian developmental biology [1], as well as heterotopic and ectopic bone formation [15]. Consider, for example, the morphogen, BMP4, known to play a critically important role in osteoinduction and osteogenesis. In addition to influencing bone development, it has been shown to participate in embryonic specification of ventral mesoderm and the early commitment to a hematopoietic fate [49, 50]. In the adult setting, when BMP4 is genetically manipulated to impair its activity, the hematopoietic niche is disrupted, disrupting with the maintenance of HSCs in the bone marrow [51].

**Perivascular Niche**

Beyond the endosteal niche, the perivascular niche is a distinct site within the bone marrow that has also been implicated in HSC maintenance and regulation [31, 52]. With the advancement in imaging technology and genetic tools that enable lineage tracing experiments and cell-type specific genetic
modulation, defining and characterizing the HSC and hematopoietic progenitor niche or niches has become ever more precise. Conditional deletion of stem cell factor (SCF), a long established regulator of HSC maintenance in the bone marrow [53, 54], from distinct cell types within the bone marrow demonstrated that ablation of SCF expression in the perivascular compartment (in both perivascular endothelial and mesenchymal cells) impaired HSC maintenance in the bone marrow. However, deletion of this canonical HSC supporting factor from osteoblast cells had no effect on the bone marrow's ability to maintain HSCs [55].

To gain deeper insight into the differing roles of the unique hematopoietic niches in the bone marrow, a systematic study of another established player implicated in the support, regulation and homing of HSCs to the bone marrow, chemokine CXCL12 (or SDF-1) expression, was conducted. The analysis demonstrated that perivascular endothelial cells are important for HSC maintenance, while perivascular stromal cells play a key role in HSC and progenitor retention. Osteoprogenitors of the endosteal niche were shown to be required for the maintenance of early lymphoid progenitors, but not HSCs or myeloid-erythroid progenitors [32]. This work underscores the complexity and multiplicity of critical microenvironments or niches within the bone marrow that, together, tightly regulate the hematopoietic system.

Given the interdependence of distinct niches within the bone marrow required to successfully support the continuum of the hematopoietic system, employing a reductionist approach and recreating individual niches or elements
of defined niches in isolation from the complex bone marrow milieu would inevitably fail to recreate all functions of the bone marrow, especially the delicate process of HSC hematopoietic progenitor maintenance and regulation. Therefore, de novo formation and true recapitulation of natural bone marrow is essential to fully harness the power of the bone marrow as the home of hematopoietic system.

Our aim is to combine microsystems and tissue engineering strategies to produce bone that contains the full complexity of the bone marrow niche. By designing a biocompatible implantable device to serve as both a container and mold for the bone inducing materials in vivo, we intend to influence the direction of cellular ingrowth into the material and impose structural constraints that shape the architecture of the resulting bone. Furthermore, the implantable device will facilitate intact removal of the tissue construct for maintenance in vitro. We believe that this systems-level approach to tissue engineering will enable the creation of an autonomous, miniaturized bone marrow construct to enable the study of whole bone marrow and the hematopoietic system in vitro.
Methods

Engineering bone marrow

Microfabrication of PDMS devices for implantation

Demineralized bone powder (DBP) was prepared from femurs harvested from adult CD-1 mice. The femurs were washed in sterile water, extracted with absolute ethanol, and dehydrated with ether. The bones were crushed with a mortar and pestle and demineralized in 0.5 N HCl (50 mL/g) for 3 hours at room temperature. After demineralization, DBP was washed with sterile water, extracted with absolute ethanol, dehydrated with ether, and was passed through a sieve with 250 mm pores. To prepare bone inducing materials, 3 mg DBP was mixed with 30 mL solution of type I collagen gel (3 mg/mL, Cellmatrix Type I-A, Nitta Gelatin Inc.), 100 ng BMP2 (Alpha Diagnostic Intl. Inc.), and 100 ng BMP4 (Alpha Diagnostic Intl. Inc.). The mixture was placed in the central cylindrical cavity (1 mm high x 4 mm diameter) of the device (Fig. 1A, B) was fabricated from polydimethylsiloxane (PDMS) formed from pre-polymer (Sylgard 184, Dow Corning) at a ratio of 10:1 base to curing agent.

Animals

The PDMS devices were filled with the bone-inducing materials and implanted subcutaneously on the backs of 8-20 weeks old CD-1 mice (Charles River Laboratories) or C57BL/6-Tg(UBC-GFP)30Scha/J (Jackson Laboratories)
mice that express green fluorescent protein (GFP) under the ubiquitin C promoter in all cells, rendering an eBM that express GFP in all of its cells. The devices were sutured onto the surface of the muscle in the subcutaneous space on the back of a mouse under anesthesia (2-4% isoflurane). Four to 8 weeks after implantation, the engineered bone marrow (eBM) was surgically removed from the mouse for subsequent characterization.

**Histology**

Following harvest, eBM and mouse femurs were fixed in 4% paraformaldehyde for 48h at 4°C. The tissue construct was transferred into 70% ethanol for storage. Once fixed, tissues were decalcified (Richard-Allen Scientific Decalcification Solution, Thermo Scientific), embedded in paraffin, sectioned and stained with hematoxalin and eosin (H&E).

**Immunohistochemistry**

Paraffin-embedded sections were deparaffinized and re-hydrated through a combination of washes in xylene and ethanol (xylene, xylene:ethanol (1:1), 100% ethanol, 95% ethanol, 75% ethanol, 100% de-ionized water). Antigen retrieval was completed (Rodent Decloaker, Biocare Inc.) followed by blocking and staining with primary antibodies, anti-mouse CXCL12 (eBioscience) and anti-mouse CXCR4 (Novus Biologicals) for 2 hrs at room temperature. Sections were stained with horseradish peroxidase (HRP) followed by Dab betazoid (Biocare
Sections were counterstained with hematoxalin and dehydrated in a series of ethanol and xylene washes.

**Micro-computed tomography (micro-CT)**

eBM harvested from mice 4 and 8 weeks after device implantation were fixed for 48 hours in 4% paraformaldehyde (PFA) and stored in 70% ethanol at 4°C. For comparison, vertebrae were harvested from the same mice immediately following device removal and were handled in a similar fashion. Both the eBM and vertebrae were imaged (in 70% ethanol) with an XRA-002 X-Tek MicroCT system. X-ray transmission images were acquired at 55kV and 200μA and the 3D reconstructions were performed using CT-Pro (Nikon Metrology); surface renderings were generated using VGStudio Max.

**Elemental mapping**

eBM and vertebrae harvested and fixed as described for micro-CT were serially dehydrated into 100% ethanol and then embedded in Spurr's resin and sectioned at the desired imaging plane using a slow speed diamond saw. The resulting sections were polished with silicon carbide papers down to P1200, sputter coated with gold or carbon and examined using a Tescan Vega-3 scanning electron microscope equipped with a Bruker X-Flash 530 energy dispersive spectrometer (EDS). All backscattered SEM images, EDS spectra, and elemental maps were acquired at 20keV accelerator voltage. For calculating
elemental composition of both the sectioned implant and vertebra samples, 10 point spectra from the surface of each sample were acquired and the percent phosphorus and calcium content was determined by averaging the obtained values ± standard error of the mean (S.E.M).

Flow cytometry

To harvest hematopoietic cells from the eBM, the tissue construct was cut into small pieces and digested using 1 mg/mL collagenase for 30 min at 37 °C. The digested tissue was filtered to remove boney tissue. Bone marrow was harvested from mouse femurs (mBM) by flushing the marrow cavity with a 21G needle.

The hematopoietic cell composition of eBM and mBM was analyzed by multicolor flow cytometry analysis using an LSRFortessa (BD Bioscience) with antibodies directed against characteristic blood surface antigens (Tables 1, 2). Cell viability was assessed by propidium iodide (PI) staining.
Hematopoietic Stem Cell (HSC) Antibodies | Hematopoietic Progenitor Antibodies | Hematopoietic Lineage Antibodies
--- | --- | ---
Lineage Cocktail (eFluor450) (CD3, CD45R, CD11b, Ter119, Gr1) | Lineage Cocktail (eFluor450) (CD3, CD45R, CD11b, Ter119, Gr1) | Ter119 (APC-eFluor780, erythrocytes)
Sca1 (APC) | CD34 (PE-Cy5) | CD45 (Pacific Blue, lymphocytes)
cKit (CD117, APC-eFluor780) | CD135 (PE) | CD19 (APC, B-cells)
CD150 (PE) | CD150 (PE) | CD3 (APC-Cy7, T-cells)
CD48 (APC) | | Mac1 (PE, macrophages)
Gr1 | | Gr1 (APC, granulocytes)

Table 1. Fluorescently labeled flow cytometry antibodies for mouse hematopoietic cells. The fluorescent antibodies listed in the table will be used to evaluate the presence of mouse hematopoietic stem, progenitor and lineage-restricted differentiated populations within the eBM, Dexter-type culture and normal bone marrow.

Hematopoietic Stem Cell (HSC) Antibodies | Hematopoietic Progenitor Antibodies
--- | ---
HLA-ABC (Pacific Blue) | HLA-ABC (Pacific Blue)
Lineage Cocktail (FITC) (CD3, CD14, CD16, CD19, CD56) | Lineage Cocktail (FITC) (CD3, CD14, CD16, CD19, CD56)
CD38 (PE-Cy7) | CD34 (APC)
CD190 (PE) | |

Table 2. Fluorescently labeled flow cytometry antibodies for human hematopoietic cells. The fluorescent antibodies listed in the table will be used to evaluate the presence of human hematopoietic stem and progenitor cells within the eBM and dish culture.

*In vitro microfluidic culture system*

Microfluidic device

Microfluidic devices for *in vitro* culture of eBM consist of three PDMS layers separated by two porous PDMS membranes (100 mm diameter pores;
Fig. 2). Tubing is attached to the input and output channels. The input tubing is connected to a syringe pump (BS-8000, Braintree Scientific) delivering continuous flow of media (see below) to the top and bottom channels at a constant rate of 0.005 dyn/cm$^2$ (1μL/min).

**In vitro culture conditions (eBM)**

Four to eight weeks after implantation, the engineered bone marrow (eBM) was removed from the PDMS device, punctured in multiple places with a 30G surgical needle, and cultured in a microfluidic device using HSC and hematopoietic progenitor media (SFEM basal media, StemCell Technologies) containing cytokines (50 ng/mL mouse SCF, 100 ng/mL mouse IL-11, 100 ng/mL mouse Flt-3 ligand, and 20 mg/mL human LDL). For experiments conducted in the absence of exogenous cytokines (Figure 12) hematopoietic progenitor media was used supplementation of cytokines.

**Bone marrow cell culture (Dexter Culture)**

Bone marrow stromal cells were harvested from 8- to 12-week-old C57BL/6 mice, re-suspended in DMEM medium (Gibco) containing 20% FBS (Gibco), GlutaMAX (Gibco), and 100 units/mL penicillin-streptomycin (Gibco). The stromal cells were cultured on tissue culture plates (Falcon), changing the medium every other day. After the adherent monolayer became established (about 3 weeks), the cells were irradiated with 12 Gy. Bone marrow cells
harvested from femurs of C57BL/6-Tg(UBC-GFP)30Scha/J mice were cultured on this bone marrow stromal cell layer using the same culture medium as is used in the microfluidic culture.

**Human umbilical cord blood (hCB) cells**

Fresh human umbilical cord blood was processed using Ficoll-Paque to fractionate mononuclear cells from the whole blood sample by density gradient centrifugation. Briefly, hCB was layered onto the Ficoll-Paque (GE Healthcare Life Sciences) gradient and spun (400 x g for 30 minutes at 20°C), separating the mononuclear cells from the plasma, granulocytes and erythrocytes. Cells were re-suspended in SFEM basal media (StemCell Technologies) containing cytokines including Flt-3 ligand, SCF, IL-3, and IL-6 contained in a pre-mixed product (StemSpan CC100, StemCell Technologies). For storage, cells were frozen in medium supplemented with 10% DMSO.

**In vitro culture conditions (hCB)**

Engineered bone marrow (eBM) harvested 8 weeks after implantation was removed from the PDMS device. We utilized two strategies to inactive the mouse immunologic system. The first included fixation in 4% PFA for 48 hours. The second strategy involved for transferred to medium designed to support human HSCs and hematopoietic progenitors, SFEM basal media (StemCell Technologies) containing cytokines including Flt-3 ligand, SCF, IL-3, and IL-6.
contained in a pre-mixed product (StemSpan CC100, StemCell Technologies).

One hour after incubation at 37°C, the eBMs were exposed to 12Gy gamma radiation (Cs-137 source) to inactivate the mouse hematopoietic cells. After either inactivation strategy, the cells in the marrow compartment were flushed out using a 30G needle. 0.5 - 1 x 10^7 hCB mononuclear cells were seeded into the boney construct. The hCB-filled eBM was inserted into the microfluidic device for 4 or 7 days of in vitro culture.

**In vivo engraftment assay**

**Bone marrow transplantation**

Eight week old C57BL/6J female mice (Jackson Laboratories) were irradiated with 12Gy (2 doses of 6Gy separated by 2-3 hours) using a Cs-137 source. The bone marrow cells were harvested from femurs of C57BL/6-Tg(UBC-GFP)30Scha/J mice or from eBM produced in similar GFP-labeled mice after 4 or 7 days of microfluidic culture. 2.5 x 10^5 bone marrow cells were delivered by intravenous (i.v.) tail vein injection within 12 hours of lethal irradiation. Animals were maintained on an antibiotic (Baytril, Bayer Corporation) to prevent bacterial infection related to radiation-induced immunosuppression. Engraftment was measured 6 weeks and 16 weeks after transplantation in peripheral blood collected by retro-orbital bleeds and evaluated by flow cytometric analyses to assess percent engraftment and degree of contribution to differentiated blood lineages.
RESULTS

Engineering Ectopic Bone and Bone Marrow

We sought to engineer an *in vitro* system that faithfully recapitulates or mimics the complete bone marrow microenvironment containing all component hematopoietic niches. To do so, we tested the hypothesis that leveraging existing bone tissue engineering techniques and combining them with microsystems strategies, we can produce bone containing a functional marrow *in vivo* that can be explanted intact and maintained *in vitro* under microfluidic culture conditions.

In order to create ectopic bone containing marrow of a defined shape and size that is not imbedded inside tissue, we microfabricated devices using a biocompatible polymer polydimethylsiloxane (PDMS) [56], to serve as a mold inside which bone and bone marrow are induced to form (Figure 3A). Our first implant device (*implant design 1.0*) was made with a central, cylindrical cavity open on both ends. The cavity is filled with bone-inducing materials, including DBP, BMP-2 and -4, and collagen type I, an effective carrier molecule used to ensure stability and prolonged delivery of the osteoinductive properties of the BMPs in coordination with natural bone matrix [57] (Figure 3B). The filled device is sutured to the surface of the muscle in the subcutaneous space on the back of a mouse, exposing the bone-inducing materials to the underlying muscle and underside (hypodermis) of the skin (Figure 3C). Four and 8 weeks after
implantation, the devices were harvested to evaluate extent of bone and bone marrow formation.

Figure 3. Implant design 1.0: Approach to ectopic bone marrow formation. (A) A cylindrical PDMS device shows central cavity (teal) into which bone inducing materials are embedded. (B) PDMS device containing bone inducing materials, before implantation. (C) Subcutaneous implantation in mouse.

Our initial approach resulted in a limited amount of bone and bone marrow formation by 4 weeks, which showed a moderate increase by 8 weeks. Histological analysis revealed bone surrounding a small, discontinuous bone marrow compartment containing a strikingly low contribution of hematopoietic cells, compared to mouse femur bone marrow, that was heavily infiltrated by adipocytes (Figures 4, 6A). The resulting bone marrow tissue constructs looked remarkably similar to the extent and structure of ectopic bone and bone marrow previously described, including the inordinate amount of adipocytes in the marrow [18, 19].
Figure 4. Implant Design 1.0: Histological representation of bone and bone marrow 4 and 8 weeks after implantation. Histological sections of the boney constructs harvested 4 (left) and 8 weeks (middle) after implantation reveal increasing amounts of bone (orange/pink) containing marrow that is heavily infiltrated with adipocytes (round empty spaces) and low contribution of hematopoietic cells, compared to mouse femur bone marrow (right).

The presence of large numbers of adipocytes in the bone marrow can inhibit hematopoiesis [21]. Therefore, we sought to reduce adipocyte infiltration into the developing bone marrow to improve the hematopoietic composition of the marrow. In a second iteration of our design (implant design 2.0), we covered the top of the device with a layer of PDMS, sealing the central cavity to restrict access of the overlying, adipocyte-rich hypodermis to the bone inducing materials, while maintaining direct contact with the surface of the muscle (Figure 5).
Figure 5. Implant design 2.0: Improved approach to ectopic bone marrow formation. Representation of implantable PDMS device shows central cavity (teal) containing bone-inducing materials. The new design has included a layer of PDMS to seal off the top, skin-interfacing opening of the cavity. Devices are implanted subcutaneously in mice and harvested 4 or 8 weeks later.

Subcutaneous implantation of this improved PDMS device resulted in the formation of a cylindrical disk of white boney tissue containing a central, blood-containing marrow or 'engineered bone marrow' (eBM) over a period of 8 weeks (Figure 6A, 6B). Histological analysis confirmed the formation of bone by 4 weeks (Figure 6D), which appeared to be remodeled into a shell of cortical bone of relatively uniform thickness by 8 weeks (Figure 6E). While a bone marrow compartment was present 4 weeks after implantation, it contained a significant number of adipocytes. By 8 weeks, hematopoietic cells, containing relatively few adipocytes, dominated the bone marrow. Comparison of histological sections of the engineered bone marrow made in the improved device 8 weeks after implantation (Figure 6E), herein referred to as “eBM,” to an intact femur (Figure 6F) isolated from the same mouse confirmed that the morphology of the eBM was nearly identical to that of natural bone marrow.
Figure 6 – In vivo bone marrow engineering. (A) Photograph of eBMs implanted for 8 weeks immediately after harvest in PDMS device design 1.0 (left) and 2.0 (right). (B) Photographs of engineered bone marrow made in implant device design 2.0, 4 and 8 weeks after implantation. Low (left) and high (right) magnification views of histological H&E-stained sections of the engineered bone marrow formed in the (C) PDMS device design 1.0 after 8 weeks and the PDMS device design 2.0, (D) 4 and (D) 8 weeks after
implantation compared to (F) a cross-section of mouse femur bone marrow (bars, 50 and 500 μm for high and low magnification views, respectively).
Evaluation of Bone Composition

To characterize the architecture of the bone formed in our engineered tissue constructs, we utilized micro-computed tomography (micro-CT), a technique that generates a fully interactive three dimensional (3D) reconstruction of a sample based on its ability to differentially transmit X-rays, which varies as a function of average electron density. In the case of our bone samples, the X-ray signal attenuation is greatest in the zones of mineralization, and not the surrounding soft tissue.

Three-dimensional micro-CT reconstructions generated from the eBM demonstrated the formation of mineralized bone in samples harvested 4 and 8 weeks after implantation (Figure 7A). Direct comparison suggests that the bone underwent remodeling between 4 to 8 weeks of subcutaneous implantation. After 8 weeks, the boney construct displayed an ordered internal trabecular network that closely resembles the intricate architecture found in normal adult mouse vertebral bone (Figure 7A).

Energy dispersive X-ray spectroscopy (EDS) is a method that focuses an electron beam onto a sample and measures scattered or dispersed X-rays ejected from the sample that have characteristic energies based on elemental composition. Using this technique, we conducted a compositional analysis of the eBM to evaluate the calcium to phosphorus ratio, compared to a mouse vertebra control. The analysis confirmed that the eBM generated in 8 weeks is
indistinguishable from natural trabecular bone in both the distribution and composition of the mineral phase (Figure 7B, 7C). Together, these data demonstrate that our approach to subcutaneous bone tissue engineering resulted in the formation of cortical bone surrounding a trabecular bone network contained within the marrow compartment, all exhibiting a morphology and mineral composition that is consistent with native living bone.

Figure 7 - Engineered bone exhibits normal trabecular microarchitecture and mineralization. (A) 3D reconstruction of micro-CT data from eBM 4 (top) and 8 weeks (middle, bottom) following implantation. Note the ultrastructural similarities in both cortical and trabecular bone organization between the eBM 8 weeks after implantation.
and a normal mouse vertebra. (B) Compositional backscattered scanning electron (BSE) micrographs and elemental mapping using energy dispersive x-ray spectroscopy (EDS) of cross sections of a mouse vertebra (left) and an eBM 8 weeks following implantation (right). Calcium and phosphorous EDS maps reveal similar uniform mineralization in both samples (bar, 1 mm). (C) Quantitation of the EDS analysis results analyzing the relative calcium and phosphorus composition in the samples from (B) confirm indistinguishable elemental compositions in the eBM compared to living bone (all error bars = standard error of the mean (S.E.M.)).
Hematopoietic Populations in the Marrow of eBM

To rigorously characterize the hematopoietic components of the engineered bone marrow, cells were harvested from the eBM immediately after surgical removal from the mouse and analyzed by flow cytometry to evaluate the expression patterns of surface antigen unique to subpopulations or hematopoietic cell type (Table 1). The hematopoietic cell composition of the eBM was compared to the distribution of hematopoietic populations in the bone marrow of mouse femurs and peripheral blood (Figure 8).

Devices harvested 4 or 8 weeks after implantation contained all blood cell types, including HSCs (Lin⁻Sca1⁺cKit⁺, "LSK") and hematopoietic progenitor cells (Lin⁻Sca1⁺, Lin⁻cKit⁺, Lin⁻CD34⁺, Lin⁻CD135⁺) (Figure 8A, 8C), as well as both differentiated red (Ter119) and white blood cell lineages (T cells, CD45⁺CD3⁺; B cells, CD45⁺CD19⁺; myeloid cells, CD45⁺Mac1⁻/Gr1⁻) (Figure 8B, 8D).

The eBM harvested 4 weeks after implantation was only partially developed, as indicated by a lower proportion of HSCs and hematopoietic progenitor cells, compared to normal marrow (Figure 8A, 8C). However, cells harvested from the eBM at 8 weeks exhibited a distribution of HSCs, hematopoietic progenitors, and differentiated blood cells from all lineages that was nearly identical to that isolated from intact bone marrow (Figure 8). These data demonstrate that we have engineered a cylindrical disk of bone resembling...
natural cortical and trabecular bone (Figure 7), which is filled with hematopoietic cells that are nearly identical in composition to natural bone marrow (Figure 8).

Figure 8 - Hematopoietic composition of the engineered bone marrow. (A, C)
Distribution of HSCs (Lin−Sca1−cKit+, green) and hematopoietic progenitor cells (Lin−
Sca1−, cyan; Lin−cKit−, purple; Lin−CD34+, red; Lin−CD135+, blue) as quantified by flow
cytometric analysis of fresh, uncultured, mouse bone marrow (mBM) from adult femur,
eBM at 4 (eBM 4wk) or 8 weeks (eBM 8wk) after implantation, or mouse peripheral
blood (mPB) that underwent erythrocyte lysis to facilitate detection of rare HSCs. (B, D)
Distribution of erythrocytes (Ter119+, blue), myeloid cells (CD45+Mac1a+, red;
CD45^Gr1^, green; CD45^Mac1^Gr1^, purple), B cells (CD45^CD19^, cyan) and T cells (CD45^CD3^, orange) in mBM (n=6), eBM 4 wk (n=5), eBM 8 wk (n=5) and intact mPB (n=1).
Chemokines of the Bone Marrow Hematopoietic Niche

The CXC cytokine ligand 12 (CXCL12), also known as Stem Cell Derived Factor – 1 (SDF-1), is expressed on a variety of cell types in the bone marrow including, osteoblasts [47], perivascular endothelial and perivascular stromal cells [32]. Interaction with its cognate receptor, CXC chemokine receptor 4 (CXCR4), expressed on hematopoietic stem and progenitor cells is critical to the recruitment, retention and maintenance of HSCs [32, 58-60]. Given the importance of this receptor-ligand pair to the composition and function of hematopoietic cells found in the bone marrow we interrogated the expression and localization of CXCL12 and CXCR4 by immunohistochemistry. Our analysis confirmed that CXCL12 is indeed expressed in the eBM, localizing to cells including those lining the inner surface of the bone and blood vessels. CXCR4, was expressed by clusters of lymphoid cells near the endosteal and perivascular niches (Figure 9).
Figure 9 – Immunohistochemical analysis of CXCL12 and CXCR4 in eBM. CXCL12 is expressed in the (A) eBM and in (C) mouse femur (femur) by cells of the endosteal and perivascular niche. CXCL12’s cognate receptor, CXCR4, is expressed by clusters of lymphocytes in the (B) eBM and in (D) in mouse femurs (femur). Images are taken at 63X magnification.
**In vitro Maintenance of eBM**

To determine whether the hematopoietic microenvironment contained within the eBM can maintain the blood constituents of the marrow *in vitro*, we designed a microfluidic culture system to enable sterile culture of the tissue construct under continuous flow of media (Figure 10). The microfluidic device is made of three PDMS layers, including a top and bottom channel, separated by two porous PDMS membranes (100 μm diameter pores) (Figure 10A). After surgical removal of the eBM from the implanted device, the tissue was introduced into the central well (middle layer) of the microfluidic device, stacked between the porous membranes and channels, and connected to tubing to enable perfusion of the tissue from the top and bottom.

![Diagram](image)

**Figure 10** – Microfluidic culture system designed to maintain eBM *in vitro*. (A) Diagrammatic display of microfluidic designed for *in vitro* culture of eBM formed *in vivo*. The microdevice is made of three PDMS layers, including a top and bottom channel, separated by two porous PDMS membranes (100 μm diameter pores). (B) Photograph
of the bone marrow chip microdevice used to culture the eBM in vitro. (bottom; bar, 2 mm)

To test our bone marrow microfluidic culture system, eBM formed in vivo for 8 weeks was surgically removed from the mouse, punctured in multiple places with a surgical needle to permit fluid access, and cultured in the microfluidic device in serum-free medium supplemented with cytokines (Flt-3 ligand, IL-11, LDL and SCF) known to support HSCs and progenitor cells in vitro [61, 62]. *In vitro* assays currently used to test drug efficacies and toxicities on bone marrow require a minimum of 4 days of cell culture [63, 64]. Therefore, we cultured the eBM for 4 and 7 days within the bone marrow-on-a-chip system, harvested the marrow, and characterized the hematopoietic populations by flow cytometry. For comparison, bone marrow was harvested from normal mouse femurs and maintained for 4 and 7 days on a 2D stromal ‘feeder’ cell layer (Dexter-type culture), the benchmark for maintaining survival of HSCs and hematopoietic progenitor cells in vitro [8, 65].

Our analysis revealed that there was no significant difference in cell viability of hematopoietic cells in the eBM after 4 or 7 days of culture in the microfluidic device, compared to the Dexter culture (Figure 11A). Quantification of the hematopoietic populations remaining in Dexter culture after 4 and 7 days demonstrated a significant decrease ($p < 0.0005$) in the number of long-term HSCs (Lin$^-$$CD150^+$$CD48^-$ cells) and a concomitant increase ($p < 0.0005$) in the
number of hematopoietic progenitor cells (Lin\(^{-}\)CD34\(^{+}\), Lin\(^{-}\)Sca1\(^{+}\), Lin\(^{-}\)cKit\(^{+}\)) relative to the number found in normal mouse bone marrow, as previously reported [8-11] (Figure 11B, 11C). These data suggest that the long-term HSCs – the cells capable of multi-potency, long-term self-renewal and differentiation into all blood cell types – are differentiating into short-term, lineage-restricted progenitor cells in the static Dexter culture system, underscoring the inherent difficulty of maintaining HSCs in conventional in vitro culture systems.

In contrast, flow cytometric analysis revealed that the number and distribution of HSCs and hematopoietic progenitor cells in the eBM cultured for 4 and 7 days on-chip remained very similar to the proportions displayed by freshly harvested mouse femur bone marrow (Figure 11C). Importantly, the bone marrow-on-a-chip also enabled maintenance of a significantly higher \(p < 0.0005\) proportion of long-term HSCs, compared to the Dexter Culture (Figure 11B). These data suggest that the microengineered bone marrow-on-a-chip device contains a functional hematopoietic microenvironment that can be maintained for at least 7 days in culture under microfluidic flow in vitro, representing a significant improvement over conventional Dexter culture of HSC and hematopoietic progenitor cells.
Figure 11. *In vitro* microfluidic culture of eBM within the bone marrow-on-a-chip. 
(A) Viability of cells isolated from uncultured mouse femur bone marrow (mBM), femur bone marrow cultured for 4 and 7 days on a stromal cell layer in a Dexter culture (mBM, D4 & D7), eBM cells analyzed immediately after surgical removal (eBM), and eBM cells after 4 and 7 days of culture on-chip (eBM, D4 & D7). (n=7) (B) Percentage of long-term HSCs (Lin’CD150’CD48’) present in marrow after 4 and 7 days in Dexter culture (mBM, D4 & D7) versus eBM cultured in the bone marrow-on-a-chip (eBM, D4 & D7) compared with freshly harvested eBM or mBM (*p < 0.0005; n=7). (C) Proportion of HSCs (Lin’Sca1+cKit+ cells, green) and hematopoietic progenitor cells (Lin’Sca1+, cyan; Lin’cKit+, purple; Lin’CD34+, red; Lin’CD135+, blue) in the mBM and eBM populations at the time of isolation compared to 4 and 7 days of culture (n=7).

Sustained maintenance of a fully functional hematopoietic system requires the support of the bone marrow hematopoietic microenvironment in its entirety. Our assessment of the bone and blood composition of the eBM suggests that we have recapitulated the complex environment of the bone marrow. To evaluate whether or not the complement of critical inductive cues necessary to support hematopoiesis are actively produced within the eBM, we tested the ability of the eBM to support HSC and hematopoietic progenitors in the microfluidic culture system for 4 and 7 days in serum-free medium in the absence of supplemental
cytokines known to support HSC and hematopoietic progenitors.

In the Dexter culture system, removal of the HSC and progenitor-supporting cytokines from the culture media altered the distribution of HSCs and hematopoietic progenitors, reducing the overall contribution of these primitive populations (Figure 12A). This suggests that the environment offered by the Dexter culture system is unable to fully regulate self-replication, proliferation and differentiation of the primitive hematopoietic populations, leaving them vulnerable to stimulation by exogenous cytokines. In the eBM, the distribution of the HSCs and progenitor populations remained similar when maintained in the microfluidic culture system for up to 7 days with or without supplemental cytokines (Figure 12B). Taken together, these data demonstrate that the hematopoietic microenvironment of the eBM can support and regulate the hematopoietic populations in an autonomous fashion, suggesting the presence of a collection of hematopoietic niches that cooperate to regulate the hematopoietic composition of the bone marrow.
Figure 12. *In vitro* culture of eBM in the absence of supplemental cytokines. Proportion of HSCs (Lin−Sca1+cKit+, “LSK” cells, green) and hematopoietic progenitor cells (Lin−Sca1+, blue; Lin−cKit+, red; Lin−CD34+, purple) in (A) Dexter culture (mBM) and (B) eBM after 4 (D4) and 7 days (D7) in culture in the presence (+ cytokines) and absence (no cytokines) of supplemental cytokines (Flt-3 ligand, IL-11, LDL and SCF) (n=3).
In Vivo Reconstitution Assay

To unequivocally confirm the presence of functional HSCs and hematopoietic progenitor cells cultured in the bone marrow-on-a-chip, it is necessary to demonstrate self-renewal and differentiation capabilities by testing their ability to reconstitute blood formation in vivo.

To meet this challenge, we created eBM in transgenic mice that expresses green fluorescent protein (GFP) under the control of the human ubiquitin C promoter, driving the expression of the fluorescent protein in all cells, with especially high levels of expression in the hematopoietic system [66]. Bone marrow cells harvested from the GFP+ eBM maintained in culture in the microfluidic system for 4 days or from freshly harvested GFP+ mouse femurs were delivered (2.5 x 10⁵ cells / mouse) intravenously into syngeneic mice that received a lethal dose of γ-irradiation (12 Gy) to deplete hematopoietic function of the host bone marrow. Total engraftment was assessed through quantification and characterization of donor-derived (GFP+) hematopoietic cells in the peripheral blood 6 and 16 weeks after transplantation to confirm the presence of functional hematopoietic progenitor cells and HSCs from the donor sample, respectively.

Cells harvested from the eBM following 4 days in the microfluidic culture on-chip successfully engrafted in the mice at a similar rate as fresh, uncultured mouse femur bone marrow, showing 70% and 85% engraftment by 6 and 16
weeks after transplantation, respectively (Figure 13A). Furthermore, the transplanted cells from the cultured eBM populated all lineages of differentiated blood cells (Figure 13B). These data confirm the presence of functional hematopoietic progenitor and multipotent, long-term HSCs in the eBM and suggest that abundance of these repopulating cells is comparable to freshly harvested bone marrow after 4 days of culture in the microfluidic system.

![Bar charts](image)

**Figure 13.** Bone marrow transplantation to evaluate presence of functional HSCs and hematopoietic progenitors. (A) Extent of bone marrow engraftment in lethally irradiated mice transplanted with $2.5 \times 10^5$ GFP$^+$ cells from fresh mouse bone marrow (mBM) or isolated from the eBM following 4 days of *in vitro* culture on-chip (eBM D4). Engraftment is presented as percentage of GFP$^+$ cells in the lymphoid population (CD45$^+$) of peripheral blood measured 6 weeks and 16 weeks after transplantation to confirm retention of functional short- and long-term HSCs, respectively. (B) Distribution of differentiated blood cells within the engrafted CD45$^+$ population from mBM and eBM D4 transplants 6 and 16 weeks after transplantation. Differentiated cell types include T cells (CD45$^+$CD3$^+$, purple), B cells (CD45$^+$CD19$^+$, green) and myeloid cells (CD45$^+$Gr1$^+$, red; CD45$^+$Mac1$^+$, blue).
Maintenance of Human Umbilical Cord Blood in eBM

The development of an in vitro platform that can successfully maintain the mouse bone marrow microenvironment and its hematopoietic contents, including multipotent, self-replicating HSCs and progenitors, suggests the potential for a translational application as a system to culture and maintain human blood equivalents for pharmaceutical or therapeutic applications. While there have been innumerable attempts and reports of human HSC expansion in vitro, these studies typically fail to achieve the intended goal of scaling production of true, functional, HSCs (e.g. a lack robust multi-lineage engraftment [67]). Given the clinical efficacy [68] and frequency of hematopoietic stem cell transplants (HSCT) procedures (numbering over 50,000 world wide in 2006 [69] and increasing ever since) and the growing dependence on umbilical cord blood (hCB) a source, a tool that could maintain -- or even induce -- self-renewal of human hematopoietic stem cells for transplantation would be of great translational value [62]. Beyond direct clinical applications, an in vitro system that reliably recapitulates the behavior of human bone marrow may prove to be an asset for the pharmaceutical industry to facilitate reliable predictions of the efficacy of drugs for hematopoietic diseases or screen for potential bone marrow toxicity in drugs under development.

We conducted a set of preliminary experiments to test the ability of the eBM to support human HSCs and hematopoietic progenitors derived from a
source rich in these primitive populations, umbilical cord blood (hCB). Mononuclear cells were recovered from fresh umbilical cord samples by Ficoll-Paque gradient centrifugation. Following surgical removal of the eBM, we attempted two methods of eBM preparation for seeding of the hCB cells.

In the first method, we harvested the eBM after 8 weeks and fixed the tissue construct in 4% paraformaldehyde (PFA) to ensure complete inactivation of the mouse cells in order to prevent an immune reaction from being mounted by residual mouse blood. After fixation, the mouse marrow contained within the eBM was physically flushed out of the mouse bone using a 30G needle to create space to accommodate seeding of the hCB. After cell seeding into the fixed tissue, the hCB-filled eBM was introduced into the microfluidic device (Figure 10) for 4 or 7 days of culture.

**Figure 14. In vitro maintenance of human umbilical cord blood (hCB) in fixed eBM.** (A) Viability of hCB measured by propidium iodide (PI) staining after 4 and 7 days in dish culture (Dish D4 & D7) or eBM cultured in the bone marrow-on-a-chip (eBM D4 & D7) compared with freshly harvested hCB (n=5) (B) Percentage of long-term HSCs (Lin–CD38–CD90+) present after 4 and 7 days in dish culture or eBM cultured in the bone
marrow-on-a-chip, compared with freshly harvested hCB (n=5). (B) Percentage of hematopoietic stem / progenitors (Lin-CD34+) present after 4 and 7 days in dish culture or eBM cultured in the bone marrow-on-a-chip, compared with freshly harvested hCB (n=5).

Flow cytometric analysis (Table 2) of hCB following 4 and 7 days in culture demonstrated that there was no significant difference in cell viability between traditional dish culture and eBM-on-a-chip system (Figure 14A). Evaluation of long-term HSCs (Lin-CD38-CD90+) showed that the eBM conferred an advantage over traditional dish culture in maintaining these cells. However, there appeared to be significantly more stem/progenitor CD34+ (Lin-CD34+) in dish culture, compared to eBM. Considering that CD34 is a relatively promiscuous marker expressed by stem and progenitor cells, this may suggest that the bona fide HSCs are differentiating in the artificial environment offered by tissue culture plastic. Due to the difficulty of seeding the hCB into the small marrow compartment of the eBM, technical improvements are required to increase the reproducibility of the method and thus reduce variability in our results.

The second method we employed was the use of γ-radiation (12 Gy), instead of PFA fixation, to inactivate but retain live mouse cells. Following radiation, the same procedure was completed, including flushing the hematopoietic cells out of the eBM, seeding of hCB mononuclear cells, followed
by introduction into the microfluidic culture system for maintenance under constant flow for 4 and 7 days.

Using this approach, flow cytometric analysis revealed a decrease in hCB long-term HSCs (Lin-CD38-CD90+) between 4 and 7 days in traditional dish culture (Figure 15A). In the eBM microfluidic culture system, there appeared to be an initial reduction in HSCs that remained stable between 4 and 7 days in culture. Evaluation of hematopoietic stem / progenitor population (Lin-CD34+) showed an initial increase in both dish and eBM culture systems by 4 days that was reduced by 7 days in dish culture but continued to increase in the eBM (Figure 14B). Given that these preliminary results represent data from one experiment, the replication is required before conclusions can be drawn.

Figure 15. In vitro maintenance of human umbilical cord blood (hCB) on irradiated eBM. (A) Percentage of long-term HSCs (Lin'CD38-CD90') present after 4 and 7 days in dish culture (Dish D4 & D7) or eBM cultured in the bone marrow-on-a-chip (eBM D4 & D7) compared with freshly harvested hCB (n=1). (B) Percentage of hematopoietic stem / progenitors (Lin'CD34') present after 4 and 7 days in dish culture (Dish D4 & D7) or eBM cultured in the bone marrow-on-a-chip (eBM D4 & D7) compared with freshly harvested hCB (n=1).
DISCUSSION

The bone marrow-on-a-chip microtechnology described here provides an important proof-of-concept for the creation of a system that recapitulates the complex bone marrow microenvironment in its entirety that can be maintained outside of a living animal, in vitro. By leveraging microfluidic strategies, this platform enables control of the naturally existing interdependent, distinct anatomical hematopoietic niches in the bone marrow, offering a powerful tool to maintain the complete collection of hematopoietic populations in the correct proportions to model and study hematopoiesis at a tissue level. Furthermore, it may offer translational value by serving as an in vitro alternative to test and predict efficacy or toxicity of drugs targeting the bone marrow, maintenance or manufacture of blood populations for therapeutic applications, or even as an in vitro diagnostic system.

The approach described here differs from conventional tissue engineering approaches in which materials or living cells are implanted in vivo without geometric constraint, and often embedded within tissues of living animals, offering little control over the environment in which the heterotopic or ectopic tissue is induced to form. By utilizing a biocompatible polymer, PDMS [56], that is FDA-approved for use in implantable prosthetic devices in humans (e.g., breast prostheses), we have created a flexible mold that can be filled with limitless combinations of tissue-inducing biomaterials while offering control over the
localization and dimensions of the resulting tissue. Illustrated by our design modifications (implant design 1.0 vs. implant design 2.0) of the implantable PDMS device (Figures 3, 5), the ease with which the moldable material can be re-designed and tested offers a moderate throughput approach to iterative design improvements. The flexibility of the system is further enhanced by the adjustable parameters of the *in vitro* microfluidic culture system, including delivery rate and content of culture medium.

The initial design of the PDMS implant created to produce ectopic bone marrow yielded only moderate success, inducing modest bone formation surrounding a marrow cavity heavily populated with adipocytes and relatively low hematopoietic cell content, compared to mouse femur bone marrow (Figure 4). Given the negative influence adipocytes exert on hematopoiesis in the bone marrow [21], we sought to improve the design by covering the cavity containing the bone-inducing materials with a layer of PDMS. Our original hypothesis was that physical restriction of the adipocytes in the overlying hypodermis from the bone-inducing materials would reduce the number of resident adipocytes that migrated into the engineered marrow. While the second design achieved the desired result (Figure 6C, 6E), we cannot eliminate the possibility that sealing the top of the cavity effectively altered its chemical (pH, oxygen tension, etc.) and/or physical milieu and induced the infiltrating undifferentiated mesenchymal cells recruited to the osteoinductive material toward an osteoprogenitor instead of an adipocyte fate (Figure 16) [14, 70-72].
Figure 16 – Osteogenic vs. adipogenic potential of bone marrow undifferentiated mesenchymal cell. Diagram illustrating the ability of the undifferentiated mesenchymal cells from adult bone marrow to undergo differentiation into the osteogenic lineage versus the adipogenic lineage. Modified from Bianco et al. [70]

The compositional analysis of the resulting eBM generated 4 and 8 weeks after implantation in our improved device (Figure 5) suggests the formation of ectopic bone induced in the device undergoes a remodeling process between 4 and 8 weeks to produce tissue whose architectural and mineral content are nearly indistinguishable from natural bone (Figure 7). In parallel with the structural maturation of the bone between 4 and 8 weeks of implantation, the distribution of the hematopoietic content of the marrow also changed, more closely matching that of naturally existing femur bone marrow by 8 weeks (Figure 8). This ‘normalization’ of the eBM suggests that the bone and bone marrow formation in the ectopic compartment follows a coordinated process or program inherent to the collection of infiltrating niche-forming cells executed according to the dynamically changing local cues within the artificial, implanted...
device [15-17]. This observation underscores the critical importance and power conferred by the physical microenvironment or niche to an incompletely differentiated cell in regulating its ultimate lineage fate and function and supports the broadly accepted dogma that "structure equals functions." Perhaps we can now include "fate," and propose that "structure equals function and dictates fate" in the case of incompletely differentiated cells.

In the context of the eBM, our work implies that a cell or collection of cells (e.g., multipotent mesenchymal cell) originating in muscle tissue proximal to the morphogen rich bone-inducing material, moves into this defined environment and is provided with a sufficient amount of guidance or information to begin a process (differentiation, expression and secretion of bioactive molecules, recruitment of additional cells, etc.) ultimately resulting in the formation of the extraordinarily complex bone marrow niche that is nearly identical to what develops in a whole animal as a result of highly regulated developmental program.

The incredible influence that a microenvironment has on the fate and function of an incompletely differentiated cells underscores the extraordinary potential of the eBM in the context of hematopoiesis. Complete reconstruction of the tightly regulated, extremely complex milieu of natural bone marrow enables us to leverage natural biological programs that have evolved over millions of years to create the ideal environment to support the hematopoietic system, maintaining all blood populations in the correct ratios. What could possibly be
better than a nearly exact replica of its actual environment for maximal retention of function outside of a living animal?

Indeed, we found that when removed from the host animal and introduced into a microfluidic culture system, the eBM maintained the full complement of hematopoietic cells in normal proportions for at least one week in culture, including functional self-replicating, multipotent HSCs and hematopoietic progenitors cells in culture better than other in vitro methods (Figures 11, 13). Even in the absence of exogenous cytokines that are shown to be important regulators of HSCs and progenitors, the eBM alone behaved in a functionally autonomous fashion to maintain the stem and progenitor populations in normal ratios (Figure 12).

In light of the potential translational applications for the bone marrow-on-a-chip microtechnology, we attempted two strategies, chemical fixation (Figure 14) or γ-irradiation (Figure 15), to disable the mouse immunologic response to enable the use of the mouse eBM to culture human umbilical cord blood-derived mononuclear cells. Using a fixation-based protocol, we show that even in the absence of live cells, the 3D architecture of the eBM showed a trend towards a modest advantage compared to traditional to dish culture in maintaining human long-term HSCs in vitro. The very preliminary data we collected using radiation protocol suggested that over longer periods in culture, there may be a trend towards improved maintenance of HSCs and progenitors by the inactivated, live mouse hematopoietic niched, though no real conclusion can be drawn in the
absence of additional data. These methods require significant optimization to standardize the approach to reduce variability and improve the functional outcome.

The bone marrow-on-a-chip microtechnology is made possible by truly working at the intersection between of engineering and biology to leverage and improve existing strategies for new purposes. Learning from fields ranging from bone engineering, developmental, stem cell, bone and blood biology, as well as microfluidic engineering, we have combined a diverse set of techniques to take advantage of natural mammalian biology to replicate the complexity of real, functional bone marrow. Our system offers a tool to enable a degree of discovery and mechanistic understanding not otherwise possible in vitro. While much work remains to adapt the system for human-specific applications, this work offers an important first step in harnessing the power of microtechnology to influence the function and fate of hematopoietic cells.

FUTURE DIRECTIONS

By engineering whole bone marrow in vivo, we have recreated the entire hematopoietic microenvironment enabling in vitro maintenance of a functional, proportionally sound hematopoietic system. Furthermore, we demonstrate that the eBM can autonomously produces the factors necessary to support the
maintenance and function of the hematopoietic system, thus relieving the
dependence of expensive supplemental cytokines to ensure maintenance of all
hematopoietic populations, including HSCs and hematopoietic progenitor cells.
This may prove to be a major advantage, enabling functional bone marrow
studies of extended duration in vitro that would otherwise be cost-prohibitive.

The bone marrow-on-a-chip also may serve as a platform for the expansion or production of specific types of blood cells by modifying the medium and adding appropriate cytokines (e.g., HSCs \textsuperscript{10}, erythropoietin \textsuperscript{28} to form red blood cells; thrombopoietin \textsuperscript{29} to form platelets; GM-CSF to form white blood cells) and collecting cells from the outflow for off-chip use and analysis. This could eventually lead to the production of blood replacements for patients with a wide variety of hematologic diseases ranging from cancer to anemia.

It is also important to note that the ability to produce trabecular bone with architectural and compositional properties similar to natural bone offers a new way to produce bones of pre-defined size and shape, and therefore serve as a tool to study bone biology, remodeling, and bone pathophysiology in vitro.

Given the completeness of the bone marrow hematopoietic microenvironment within the eBM, the system may be useful to mimic the complex tissue-level responses of normal marrow to radiation toxicity, medical countermeasures that might protect against radiation poisoning, or other drugs under development, thus becoming a valuable in vitro replacement for whole animals. In addition, the possibility of visualizing and studying the intact bone
marrow niche in situ over time could provide entirely new ways to evaluate the effects of drugs and toxins on hematopoiesis, as well as to model complex hematologic diseases in vitro.

While we show preliminary strategies attempted to create a human model system, one could envision using alternative methods including engineering the eBM in immunocompromised mice (e.g., NOD.Cg-Prkdc^scid^ IL2rg^tm1Wjl/Sz; NSG) and replacing the mouse marrow with human hematopoietic cells. By humanizing our platform, we could conduct patient-specific drug screening to identify the most effective drug to treat a patient's hematologic disease and provide pharmaceutical companies with a tool for reliable drug screening before entering clinical trials.

The bone marrow-on-a-chip offers a powerful tool to accelerate discovery and development in a wide range of biomedical fields ranging from hematology, oncology, and drug discovery to tissue engineering.
# LIST of JOURNAL ABBREVIATIONS

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<td>Accounts of Chemical Research</td>
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BIBLIOGRAPHY


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Languages:
- English (Fluent)
- Spanish (Intermediate)
- French (Basic)

Work Experience:
- Software Engineer at XYZ Inc., 2019 - Present
- Analyst at ABC Corp., 2017 - 2019

Projects:
- Developed a web application using Python, 2020
- Created a mobile app using Swift, 2018

Technical Skills:
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- C#
- SQL

Personal Projects:
- Created a personal blog, 2021
- Participated in a hackathon, 2022