Video Article

A Three-dimensional Tissue Culture Model to Study Primary Human Bone Marrow and its Malignancies

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Abstract

Tissue culture has been an invaluable tool to study many aspects of cell function, from normal development to disease. Conventional cell culture methods rely on the ability of cells either to attach to a solid substratum of a tissue culture dish or to grow in suspension in liquid medium. Multiple immortal cell lines have been created and grown using such approaches, however, these methods frequently fail when primary cells need to be grown *ex vivo*. Such failure has been attributed to the absence of the appropriate extracellular matrix components of the tissue microenvironment from the standard systems where tissue culture plastic is used as a surface for cell growth. Extracellular matrix is an integral component of the tissue microenvironment and its presence is crucial for the maintenance of physiological functions such as cell polarization, survival, and proliferation. Here we present a 3-dimensional tissue culture method where primary bone marrow cells are grown in extracellular matrix formulated to recapitulate the microenvironment of the human bone (rBM system). Embedded in the extracellular matrix, cells are supplied with nutrients through the medium supplemented with human plasma, thus providing a comprehensive system where cell survival and proliferation can be sustained for up to 30 days while maintaining the cellular composition of the primary tissue. Using the rBM system we have successfully grown primary bone marrow cells from normal donors and patients with amyloidosis, and various hematological malignancies. The rBM system allows for direct, in-matrix real time visualization of the cell behavior and evaluation of preclinical efficacy of novel therapeutics. Moreover, cells can be isolated from the rBM and subsequently used for *in vivo* transplantation, cell sorting, flow cytometry, and nucleic acid and protein analysis. Taken together, the rBM method provides a reliable system for the growth of primary bone marrow cells under physiological conditions.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50947/>

Introduction

Tissue culture was developed to study cell behavior in a controlled environment to minimize the systemic variability when comparing various processes in intact organisms. This method was first established in the early 1900^{1,2} and refers to a technique where tissue explants were cultured *ex vivo* in a glass dish. In the mid-1900s the system was adapted to grow dispersed cells rather than fragments of intact tissues, and the terms 'tissue culture' and 'cell culture' became synonymous³. In such conventional cell culture systems cells are grown on the surface of the tissue culture plastic overlaid with growth medium supplemented with various growth factors. Two types of cultures have emerged based on the adhesion capacity of various cells: adherent cell culture, where cells attach and spread on the tissue culture plastic and nonadherent cultures, where cells are propagated in suspension. Since the early days of cell culture, multiple immortal cell lines have been created, such as HeLa⁴, the first human cancer cell line. These cell lines have the capacity to proliferate indefinitely in cell culture, and most do not require any special treatment to maintain viability.

The reductionist approach of the original cell culture methods was designed to simplify the system as much as possible by including only the bare minimum components required to sustain cell viability and proliferation. However, simplified cell culture approaches fail to support *ex vivo* most primary human cell types with finite life-span. Therefore, new culture systems are being designed to approximate the tissue microenvironment as closely as possible, thus allowing cell explants to grow under physiological conditions. In contrast to the conventional/reductionist cell culture approaches, 3-dimensional (3-D) culture systems are now becoming a preferred method to efficiently culture various human cell lines and primary cells to subsequently study mechanisms involved in health and disease, in the context of a supportive microenvironment. Such 3-D systems are usually set up using reconstructed matrices and/or medium supplemented with growth factors, in order to recapitulate the tissue microenvironment to study cell types of interest. The first of these 3-dimensional (3-D) culture models was developed to study mammary gland development. To provide the cells with native conditions mammary epithelial cells were embedded in Matrigel, collagen IV and laminin-rich source of extracellular matrix (ECM), and overlaid with growth medium. Under such conditions, the mammary epithelial cells formed clusters resembling the mammary acini, and upon stimulation with lactogenic hormones, these acini secreted

casein, and other milk proteins, into the hollow lumina of the acini-like structures. Casein secretion was not observed in standard cultures even after addition of prolactin⁵, further emphasizing the role of microenvironment in preserving the morphological and phenotypic characteristics of cells. Another demonstration of the loss of normal cellular function when cells are taken out of the context of their physiological microenvironment is a demonstration that without supportive microenvironment, keratinocytes fail to form stratified epidermis⁶. A number of other 3-D models have been created to allow *ex vivo* propagation of primary cells^{7,8}.

The crucial role of microenvironment in cell behavior was elegantly demonstrated in a study where mammary epithelial cells formed "inside-out" acini when cultured in collagen I matrix, compared to the correctly polarized acini that were formed in Matrigel. This loss of proper morphology was reverted when laminin-producing myoepithelial cells were added to the collagen I cultures⁹. Moreover, correct microenvironment is required for the accurate genotype manifestation. Grown in a dish, MCF7 breast cancer cells transfected with a cell-cell adhesion molecule CEACAM1 behave exactly the same as the untransfected CEACAM negative cells. However, when cultured in Matrigel, in contact with ECM, wildtype MCF7 form tumor-like structures while MCF7 cells transfected with CEACAM1 revert to a normal phenotype and form acini with hollow lumina, as has been established with nonmalignant mammary epithelium¹⁰. Similarly, blocking β 1-integrin in breast cancer cells does not change their behavior under standard culture conditions, but the same experiment performed in 3-D cultures demonstrates that blocking β 1-integrin reverts malignant cells to a normal phenotype¹¹. Therefore, tissue microenvironment is not only required to maintain cell viability, but also to retain proper cell function.

In addition to providing a system where cell behavior can be studied under physiological conditions, 3-D cultures function as robust and reliable medium for preclinical testing of novel therapeutics^{8,12,13}. Culturing cells in 3-D allows screening of investigational compounds under the conditions of environment-mediated drug-resistance¹⁴, where the contribution of cell-cell and cell-ECM adhesion could be assessed. Furthermore, off-target toxicity of new compounds can be ascertained by incorporating multiple cellular compartments of various tissues. Such screens can be performed more rapidly and are more cost-effective than the comparable studies *in vivo*⁸.

Here we present a setup of a 3-D model of reconstructed bone marrow (rBM) where normal and malignant bone marrow (BM) cells proliferate *ex vivo* in a system closely mimicking the microenvironment of the human BM. Previous attempts at growing primary human BM cells in 2-D cultures, liquid or adherent cocultures with various components of BM stroma, or 3-D, semi-solid agar cultures have met with limited success due to their inability to supply the cells with the components of tissue microenvironment¹⁵⁻¹⁸. In these systems primary BM cells had poor viability and failed to proliferate *ex vivo*. Another system where human BM progenitor cells are grown in spheroid cocultures with BM stromal cells is a 3-D system where hematopoietic stem cell viability and proliferation was sustained for at least 96 hr¹⁹. However, although highly beneficial to the understanding of the hematopoietic progenitor biology, this system does not faithfully recapitulate the microenvironment of the BM owing to the absence of ECM components, therefore, limiting its usefulness. The rBM model described here presents a comprehensive system where both cellular and extracellular compartments of the human BM are reconstructed *in vitro*. We show that rBM cultures can support *ex vivo* growth of normal human BM cells, as well as cells isolated from patients with various hematological disorders.

Protocol

1. Advance Preparation

1. Keep sterile serological pipettes and pipette tips at 4 °C.

Troubleshooting: Matrigel gels at room temperature (RT); using cold pipettes and tips will prevent Matrigel from gelling during the culture setup.

2. Thaw a bottle of Matrigel at 4 °C overnight.

2. Preparation of Reagents

1. Preparation of fibronectin stock solution: Make a 1 mg/ml stock solution of fibronectin by dissolving 100 mg human fibronectin in 100 ml of distilled water. Once the fibronectin has dissolved, sterile filter, aliquot, and store at 4 °C for up to 6 months.

Troubleshooting: If fibronectin doesn't dissolve quickly incubate the solution for a few minutes in a water bath set at 37 °C.

2. Preparation of 10x PBS: Dissolve 80 g sodium chloride NaCl, 14.4 g Na₂HPO₄, 2 g KCl and 2.4 g KH₂PO₄ in 1 L of distilled water. Set the pH to 7.4, sterile filter, and store at 4 °C for up to 6 months.
3. Preparation of neutralization buffer²⁰: Make a solution containing 100 mM HEPES in 2x phosphate buffered saline (PBS) using 1 M HEPES and 10x PBS stock solutions. Adjust the pH of the solution to 7.0. Store at 4 °C for 2-3 months.
4. Preparation of 2 mg/ml solution of rat tail collagen type I: Make a 2 mg/ml collagen I solution in the neutralization buffer (step 2.3). Vortex the solution at low speed and mix gently by pipetting up and down. As this collagen is very viscous, avoid generation of air bubbles while pipetting. It is recommended to prepare collagen I solution fresh every time. Keep the solution on ice until use.
5. Preparation of endosteal coating (rEnd): Mix 1 mg/ml fibronectin stock with 2 mg/ml collagen I stock (steps 2.1 and 2.4) to a final concentration of 77 µg/ml and 29 µg/ml respectively, in 1x sterile PBS. Mix and store at 4 °C for up to 1 month.
6. Preparation of reconstructed bone matrix (rBM):
 1. Precool 1.5 ml microcentrifuge tubes on ice. Keep all matrix solutions on ice at all times.
 2. Mix 4 parts Matrigel (Matrigel concentration varies from lot-to-lot, but the variations were found to be negligible), 2.5 parts of 1 mg/ml fibronectin and 1 part of 2 mg/ml collagen I on ice by first pipetting Matrigel into the tube using cold pipette tips and then add fibronectin and collagen I. Matrigel will start solidifying at temperatures above 4 °C, so work quickly while pipetting Matrigel. Mix the matrix very gently by pipetting up and down, avoid introducing bubbles. Keep the tubes on ice while mixing the rBM.
7. Preparation of bone marrow growth medium (BMGM): Make 500 ml of BMGM containing 6.2 x 10⁻⁴ M CaCl₂, 10⁻⁶ M sodium succinate, 10⁻⁶ M hydrocortisone, 20% human plasma (normal or malignant collected from healthy donors or patients), and 1% penicillin/streptomycin in

RPMI-1640, CaCl_2 , sodium succinate, and hydrocortisone supplements can be stored at -80°C for >6 months as 1,000x stock solutions. When growing cell lines, fetal bovine serum (FBS) can be substituted for human plasma.

8. Preparation of 10% neutral buffered formalin (NBF): Add 37% formaldehyde stock solution to 1x PBS at 10% v/v. Mix well and store at room temperature for 2-3 weeks protected from light.
9. Preparation of 1% bovine serum albumin (BSA): Weigh appropriate amount of BSA and dissolve it in 1x PBS. Store at 4°C and use within 1 week. BSA solution tends to get contaminated if kept refrigerated for longer periods. Alternatively, BSA solution can be aliquoted and frozen at -20°C for long-term storage. Avoid freeze-thaw cycles.
10. Preparation of cell recovery solution (CRS): Make 5 mM EDTA, 1 mM sodium vanadate (Na_3VO_4) and 1.5 mM sodium fluoride (NaF) solution in 1x PBS. Sterile filter and store at 4°C up to 6 months.

3. Embedded 3-D Culture of Nonmalignant and Malignant Human BM Cells

1. Purify primary BM mononuclear cells by Ficoll-Plaque gradient centrifugation per manufacturer's instructions.

Optional step: cells can be labeled with $0.25\ \mu\text{M}$ carboxyfluorescein diacetate, succinimidyl ester (CFSE) per manufacturer's instructions to follow cell proliferation throughout the culture period.

Troubleshooting: If cells die after CFSE staining, titrate the amount of CFSE as the concentration depends on cell type; $0.25\ \mu\text{M}$ works well for primary BM mononuclear cells.

2. Add $65\ \mu\text{l}$ of rEnd solution into each well of a 48-well tissue culture treated plate. Spread the solution evenly to cover the entire surface of the well and incubate for >30 min at RT.
3. Prepare the cell suspension at a density of 0.5×10^6 cells in $10\ \mu\text{l}$ of 1x PBS per well of a 48-well plate. In a 1.5 ml microcentrifuge tube mix the cell suspension with $100\ \mu\text{l}$ of rBM matrix per well. Mix the cells and rBM matrix by gently pipetting up and down; avoid introducing bubbles. Keep the cell/matrix mixture on ice to avoid gelling of the matrix.

Note: the rBM system is fully scalable; to set up the system in a non-48-well format, adjust the amounts of all reagents (matrices and medium) based on the surface area of the plate used. Scale the number of cells to be plated accordingly.

4. Aspirate the remaining rEnd and add the cell/matrix mixture into the center of a well. Quickly, spread the cell/matrix mixture to cover the entire surface of the well evenly. Place the plate for 30-60 min in 37°C , 5% CO_2 tissue culture incubator to allow the matrix to solidify. Do not shake or disturb the plate during incubation.

Troubleshooting: To prevent uneven distribution of cells in rBM, mix well prior to plating. Mix after every 5th well plated to avoid cells settling to the bottom of the tube. Once plated, cells do not sink to the bottom of the well due to surface tension in the matrix.

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5. Prewarm BMGM in a water bath set to 37°C .
6. After 30 min, check to see that the matrix has set properly; it will form a soft gel like layer that does not move when the plate is tilted. Overlay the cell/matrix layer with 1 ml of warm BMGM. To avoid tearing of the matrix pipette the medium very gently (dispensing drop-by-drop) using a serological pipette. Place the assembled rBM culture into a 37°C , 5% CO_2 tissue culture incubator and culture for the desired time period.

Note: cell viability is maintained for at least 30 days without changing medium; if medium change is required only change 50% of medium each time.

Critical step: It is crucial that the medium is not cold as cold temperature might disturb the already set matrix. Take care not to squirt the medium on top of the matrix as it might break the soft matrix layer.

Troubleshooting: If cell viability in the rBM system is low, cell density may have to be determined experimentally when working with cells other than primary mononuclear cells from BM aspirates. When working with cell lines, decrease the cell number 10-fold, as immortalized cell lines proliferate faster than primary cells.

4. 'In-matrix' Imaging

1. Light microscopy

1. Image the cultured cells directly in rBM using bright field, differential interference contrast (DIC), confocal or any other imaging techniques that allow long-distance imaging as the matrix is approximately 1 mm thick. Use the Z-stack feature available on many of the microscopes to image the entire culture from top to bottom.

2. Immunofluorescence staining

Tip: For the immunostaining protocol below avoid aspirating staining/washing solutions, instead tilt the plate and remove solutions using a pipette.

1. Remove the BMGM by using a pipette and wash 2-3x with $100\ \mu\text{l}$ /wash/well of RT 1x PBS. Make sure that enough PBS is used to cover the entire surface of the well.
2. Fix cells in matrix by adding $100\ \mu\text{l}$ /well (for a 48-well plate) of 10% NBF for 15 min at RT. Remove NBF and wash cells twice with $100\ \mu\text{l}$ /wash/well of RT 1x PBS.

Note: Avoid using cold NBF as it may liquefy the matrix.

3. Remove PBS, add 1% BSA for 1 hr at RT or at 4°C overnight in order to block nonspecific binding of antibodies.

4. Prepare primary antibody solution in 1% BSA at the required dilution. Dilution will vary for each antibody, check with the manufacturer or titrate to determine the optimal antibody concentration. Add 100 μ l/well of primary antibody solution and incubate according to the manufacturer's instructions.

Critical step: All incubations involving antibodies conjugated to fluorophores or fluorescent dyes should be performed in the dark.

5. Remove primary antibody solution and wash 3x with 1x PBS for 5 min.
6. For indirect immunofluorescence staining, incubate with a secondary antibody conjugated to fluorescent probe for 1 hr at RT. Dilute the antibodies in 1% BSA at concentration suggested by the manufacturer.
7. Remove the secondary antibody solution and wash 3x with 1x PBS for 5 min.

Troubleshooting: To avoid high background during imaging, titrate the fluorescently conjugated secondary antibodies and use the lowest concentration that provides adequate signal. If high background is still an issue, increase the number and the duration of wash steps, or use directly conjugated primary antibodies, and skip step 4.2.6.

8. To stain the nuclei add 4',6-diamidino-2-phenylindole (DAPI) nuclear staining solution, at the dilution suggested by the manufacturer, and incubate for 7-10 min at RT.
9. Remove nuclear staining solution and wash 2x with 1x PBS for 5 min.
10. Remove the PBS from the last wash and add a drop of mounting medium (regular set), on the sample to preserve fluorescence. Image using appropriate excitation/emission settings on a fluorescent or confocal microscope.

Note: Imaging should be done within 2 days of staining to avoid degradation of matrix and loss of rBM integrity and loss of fluorescent signal. If not imaged immediately, stained samples should be kept at 4 °C, protected from light, until imaging.

5. Isolation of Cells from rBM

1. Remove medium gently without disturbing the matrix layer. Do not aspirate.
2. Wash cells 2x with sterile 1x PBS. Do not aspirate.
3. Add 0.5 ml of ice-cold CRS to each well and remove the entire matrix layer along with the cells, by vigorously pipetting up and down. Collect the cells, matrix, and CRS in a 1.5 ml microcentrifuge tube. Place on ice.
4. Add an additional 0.5 ml CRS to the same well and collect all remaining material. Transfer to the same microcentrifuge tube.
5. Vortex the mixture of cells, matrix, and CRS and incubate on ice for 1hr. Vortex cells every 15 min to facilitate release from the matrix.
6. Check the mixture after 1 hr, no visible clumps of matrix should be present.

Troubleshooting: if clumps of matrix are still visible after 1 hr, transfer cells/matrix/CRS mixture to a bigger tube, add additional 2-5 ml of CRS, and incubate for an additional 30-60 min.

7. Centrifuge cells in CRS at 1,000 rpm for 5-10 min at 4 °C. Remove the supernatant and resuspend the cell pellet in 1 ml of cold 1x PBS.
8. Centrifuge at 1,000 rpm for 5-10 min and remove the supernatant. Repeat the PBS wash one more time.
9. The second PBS wash completes the recovery of cells from matrix and isolated cells can be used for any downstream applications such as DNA/RNA/protein isolation, flow cytometry, *in vivo* transplantation, etc.

Representative Results

Since primary bone marrow cells fail to proliferate under standard tissue culture conditions, the rBM system was created to closely mimic the bone microenvironment, providing a system to culture and expand BM cells *ex vivo*. The major components of the BM extracellular matrix, fibronectin, collagen I, collagen IV, and laminin²¹, were incorporated into rBM matrix to provide the structural scaffold to this 3-D culture. Endosteum, the interface between the bone and the BM, rich in collagen I and fibronectin, was reconstructed by coating the surface of a tissue culture dish with these proteins. Crosstalk between multiple cellular compartments within the BM contributes to the overall homeostasis of the tissue. To ensure that none of the cellular components are left out, the rBM culture was set up using unfractionated mononuclear cells from the human BM needle aspirate biopsies. To ensure that the system is supplied with hormones, growth factors, and cytokines present in the circulatory environment, the culture medium was supplemented with human plasma corresponding to the specimen being cultured (*i.e.* plasma from normal donors was added when nonmalignant BM cells were placed in rBM, while plasma from patients was added to the cultures of malignant cells) (**Figure 1**).

BM and blood samples from healthy donors and patients with various hematological malignancies were collected after approval from Purdue University and University of Alberta Institutional Research Boards and after written informed consent in accordance with the Declaration of Helsinki (**Table 1**). Mononuclear cells isolated from BM biopsies proliferate and maintain their viability in rBM system for up to 30 days (**Figure 2**). The cell concentration of 0.5×10^6 cells/100 μ l of rBM matrix has been determined to be the optimum density for primary BM cells. Going beyond this density overcrowds the system decreasing cell viability and proliferation rates over time. A lower density has also been shown to be detrimental to the overall health of the system as cell-cell contacts have proven vital for a robust cell growth. Growth within the rBM can be followed after labeling cells with carboxyfluorescein diacetate, succinimidyl ester (CFSE). Fluorescence intensity of CFSE halves with each cell division, thus cell proliferation can be tracked by microscopy or flow cytometry over time. Moreover, the cellular compartments within the rBM culture are maintained in the same proportions as were present in the biopsy samples⁸. In addition to the cells of the hematopoietic lineages, stromal compartments exhibit robust growth in rBM. Alkaline phosphatase expressing osteoblasts capable of mineralizing calcium, tartrate resistant acid phosphatase staining osteoclasts, oil red positive adipocytes, and fibronectin producing stromal cells are found at the endosteum/BM interface of the rBM system (**Figure 3**).

Taken together, our data demonstrate that the rBM model provides a first *ex vivo* system where primary human BM cells from healthy donors and patients with various hematological malignancies such as leukemia, lymphoma, and multiple myeloma can be sustained and expanded for up to 30 days. rBM provides a comprehensive model to study cell behavior under physiological conditions in the context of the native BM

microenvironment. This system has been used by our laboratory to identify the phenotype of the multiple myeloma cancer stem cells and to study the interactions between the malignant cells and the BM extracellular matrix as well as the cross-talk between the malignant cells and the BM stroma.

Diagnosis	Cell type	Survival	Proliferation
Nonmalignant	BM	Moderate	Moderate
Amyloidosis	BM	High	High
Monoclonal gammopathy of undetermined significance	BM	High	High
PBMC	No	No	
Plasmacytoma	BM	High	High
Smoldering multiple myeloma	BM	High	High
Multiple myeloma	BM	High	High
PBMC	No	No	
MB	High	High	
Plasma cell leukemia	BM	High	High
Waldenstroms macroglobulinemia	BM	High	High
Acute myelogenous leukemia	BM	High	High
Acute promyelocytic leukemia	BM	High	High
PBMC	No	No	
Acute lymphocytic leukemia	BM	Moderate	Moderate
Chronic myelogenous leukemia	BM	Moderate	High
Chronic lymphocytic leukemia	BM	Low	Slow
PBMC	No	No	
Non-Hodgkin's lymphoma	BM	Moderate	Slow
PBMC	No	No	

Table 1. Survival and proliferation of various specimens cultured in rBM. Bone marrow mononuclear cells (BM), peripheral blood mononuclear cells (PBMC), or mobilized blood samples (MB) from healthy donors and patients diagnosed with various malignancies were cultured in rBM. The table lists all specimen types that were cultured in rBM along with the cell type (*i.e.* BM, PBMC, or MB). The extent of cell survival and proliferation in rBM are noted. While BM and MB exhibit robust growth in rBM, PBMCs isolated from patients with various malignancies were not viable in rBM.

Figure 1. Schematic representation of rBM setup. rBM consists of two phases: 1) the endosteal coating (fibronectin, collagen I (CI)) and 2) rBM matrix (fibronectin, collagen I (CI), collagen IV (CIV) and laminin). **Note:** collagen IV and laminin are the major components of Matrigel, thus no separate addition of these proteins is required. These phases are set up in two steps by overlaying the rBM matrix/cell mixture on top of the thin coating of endosteal matrix. To follow cell proliferation over time, cells can be labeled with CFSE prior to mixing with the rBM matrix. Over the duration of the culture, cells can be visualized by microscopy and cell migration can be followed in real time using a microscope equipped with a temperature/CO₂ controlled chamber. After 14-30 days in culture cells can be isolated from the matrix and subjected to a variety of downstream analyses, including both *in vitro* and *in vivo* procedures. [Click here to view larger image.](#)

Figure 2. Time course of self-segregation of cellular compartments in rBM. BM cells were grown in rBM for the indicated number of days. Stromal elements become evident by day 14, but can be detected as early as day 5 in rBM. Over the course of the culture period cells can be seen migrating through rBM matrix and aggregating into distinct clusters. Masses of malignant cells expand over time (compare cluster sizes between day 9-30). Representative view of the culture at each time point was captured using bright field microscopy on an inverted microscope (magnification: 200X). [Click here to view larger image.](#)

Figure 3. Stromal compartments are 'supplied' within the rBM. To evaluate the composition of the stromal components that outgrow in rBM, cells at the transition between rBM matrix and endosteal coating were evaluated for fibroblast, osteoblast, adipocyte, and osteoclast-specific morphology and marker expression. **(a)** To detect fibroblasts, cells were stained for fibronectin (green), actin (red), and nuclei (blue) and imaged on a confocal microscope (magnification: 200X). **(b)** Cells with stromal morphology were shown to be preosteoblasts with high levels of alkaline phosphatase activity **(b.1)** and capable of mineralizing calcium as determined by Alizarin Red staining **(b.2)**. **(c)** Cells with a visible presence of lipid droplets were identified as adipocytes based on positive staining with Oil Red **(c.1)**. **(d)** Cells with occasional multiple nuclei were recognized as preosteoclasts and were positive for tartrate resistant acid phosphatase (TRAP) **(d.1)**. Bright field and color (nonfluorescent) images were acquired on an inverted microscope equipped with a color camera (magnification: 200X). [Click here to view larger image.](#)

Discussion

The rBM model provides a comprehensive system where cellular composition of the BM is maintained *ex vivo* for up to 30 days. BM cells grown in rBM self-stratify according to their function closely mimicking the BM architecture found *in vivo*. Moreover, this is the first system that allows for the expansion of the multiple myeloma clone⁸. The most critical steps for ensuring the robust growth of BM cells and the overall success of the culture are: 1) to obtain a healthy population of the BM cells with >70% viability and mostly free of red blood cells, 2) to ensure that the matrix components are mixed well and that the cells are distributed uniformly throughout the matrix, and 3) to take care not to damage the matrix when growth medium is added to system. To ensure the robust proliferation of malignant cells, growth medium should be supplemented with human plasma matching the diagnosis of the patient whose cells are being cultured. One limitation of the rBM system that was noticed is its inability to support purified populations of CD34+ hematopoietic stem cells, CD20+ B cells, and CD138+ plasma cells without the stromal compartment of the BM.

The rBM system is highly versatile and can be modified in many ways to best suit the goals of specific studies. Endothelial compartment can be added to the system to mimic the vascularization of the BM and additional extracellular matrix components can be supplied to bring the matrix composition as close as possible to the *in vivo* make-up of the BM and the matrix concentrations can be adjusted to reflect the disease states where the concentrations of individual matrix components may be altered²¹. In addition to culturing primary cells, rBM system is suitable for coculturing various cell lines to study cell-cell interactions between specific cell populations. For example, the system can be set up as a coculture where stromal cells are plated over the endosteal coating, hematopoietic cells are added on top of the stromal cells, and the entire stromal/hematopoietic coculture is overlaid first with the rBM matrix and then with the growth medium. Also useful, could be the modifications of the medium composition to include plasma from various sources or medium conditioned by stroma to study how soluble factors affect cell behavior¹². Cytokines, growth factors, and hormones can also be added to the growth medium, however, care should be taken not to replace all growth medium in the culture at once as growth sustaining factors that were secreted by the cells in rBM may be lost, thus, affecting the overall viability of the system. We suggest replacing up to 50% of the medium at a time.

The rBM system can be used to study both cell behavior (*i.e.* proliferation, viability, migration, *etc.*) and to assess the potency and off-target effects of novel therapeutics^{8,12}. The system is set up to recapitulate the tissue microenvironment, thus, the efficacy of investigational drugs can be evaluated under the conditions of environment-mediated drug-resistance¹⁴. Using live-cell real-time microscopy, cell viability over the course of treatment can be assessed by live/dead staining and the affect of treatment on various organelles can be evaluated using multiple commercially available organelle-specific dyes that readily penetrate the rBM matrix and are taken-up by the cells without generating significant background that could affect imaging.

The rBM system provides a highly versatile and adaptable model to study BM disorders. The benefit of such 3-D culture method compared to the standard 2-D cultures is in the ability to study cell behavior in the context of the tissue microenvironment ensuring that the effects of the multiple interactions are not missed when cells are isolated from their physiological environment and placed in a plastic dish. The appeal of the rBM system over *in vivo* models lies in the transparency of the system where the interactions that cannot be seen *in vivo* due to the limitations of imaging techniques could be easily dissected. The rBM system provides a medium for easy manipulation of components while maintaining the overall tissue composition, thus allowing the researcher to dissect the molecular mechanisms under investigation. Moreover, the rBM system has been validated as a cost-effective way, compared to *in vivo* testing, to conduct preclinical studies of novel therapeutics^{8,12}. Taken together, the rBM model provides a system where many aspects of tissue biology can be investigated *ex vivo* using primary specimens.

Disclosures

The authors declare no competing interests.

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