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Bone Marrow Microenvironment Modulation of Acute Lymphoblastic Leukemia Phenotype

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Abstract

Acute lymphoblastic leukemia (ALL) treatment regimens have dramatically improved the survival of ALL patients. However, chemoresistant minimal residual disease (MRD) that persists following cessation of therapy contributes to aggressive relapse. The bone marrow microenvironment (BMM) is an established “site of sanctuary” for ALL as well as myeloid lineage hematopoietic disease, with signals in this unique anatomical location contributing to drug resistance. Several models have been developed to recapitulate the interactions between the BMM and ALL cells. However, many *in vitro* models fail to accurately reflect the level of protection afforded to the most resistant sub-set of leukemic cells during co-culture with BMM elements. Pre-clinical *in vivo*

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models have advantages, but can be costly, and are often not fully informed by optimal *in vitro* studies. In the current report we describe an innovative extension of 2D co-culture wherein ALL cells uniquely interact with bone marrow derived stromal cells. Tumor cells in this model bury beneath primary human bone marrow derived stromal cells or osteoblasts, termed “phase dim” (PD) ALL, and exhibit a unique phenotype characterized by altered metabolism, distinct protein expression profiles, increased quiescence, and pronounced chemotherapy resistance. Investigation focused on the PD subpopulation may more efficiently inform pre-clinical design and investigation of MRD and relapse that arises from BMM supported leukemic tumor cells.

Keywords

Model; Bone Marrow Microenvironment; Leukemia; Chemoresistance; Co-culture

Introduction

The bone marrow microenvironment (BMM) is a well-established “site of sanctuary” in a host of malignancies, with the most common being of hematopoietic origin[1–8]. In leukemia, the BMM serves as the site of initiation and progression of disease. The BMM is also the most common site of relapse, where leukemic cells respond to signals that are critical for the support of “healthy” steady-state hematopoiesis[2,9,10]. Quiescence, metabolism, and survival pathways are all influenced by the BMM and are pathways known to be co-opted by leukemic cells in the marrow niche to promote treatment resistance[5,7,11]. Studies from many laboratories have furthered our understanding of the interplay between leukemic cells and the BMM, however, relapse of disease continues to be a clinical challenge.

A number of models have been employed to recapitulate the interactions between the bone marrow niche and leukemic cells. *In vivo* murine models have provided insight and have become standard pre-clinical models in which to test novel therapeutic strategies[12–14]. While *in vivo* models define the gold standard they are labor intensive, time consuming, and costly to test hypotheses related to relapse of disease. Also, while the BMM can be effectively imaged during disease progression or treatment response, sequential sampling of tumor recovered from the niche is only achievable upon termination of experiments, resulting in evaluation of snapshots in time. Often, ongoing analyses are limited to peripheral circulating tumor that does not reflect the most treatment-resistant subpopulation of interest. Standard 2D *in vitro* models, while lacking the complexity of the *in vivo* microenvironment, provide an alternative means to interrogate tumor interactions with the microenvironment. Several groups have demonstrated that 2D co-culture with primary human bone marrow stromal cells (BMSC) and osteoblasts (HOB) protect human leukemic cells from chemotherapy induced death[2,6,8,11,15]. However, standard *in vitro* models lack the ability to predict long term survival of sub-sets of resistant leukemic cells, and as a result, are not ideal for evaluation of mechanisms that underlie MRD.

Studies including co-culture of healthy hematopoietic stem cells with mesenchymal stromal cells (MSC) revealed that co-culture models exhibit a more dynamic nature than was

previously appreciated. Hematopoietic cells interacted with MSCs in three distinct spatial compartments[16]. The subpopulations included uniquely identifiable suspended (S), phase bright (PB), or phase dim (PD) tumor cells when evaluated by light microscopy. Differences in the hematopoietic stem cell phenotype correlated with location of the hematopoietic cell relative to adherent MSC. Of particular relevance to the current study was the observation that the “phase dim” (PD) population of hematopoietic cells that buried beneath the MSC monolayer was immature and quiescent, two characteristics that have been associated with chemotherapy resistance[16,17]. In addition, it has previously been described that tumor cells closely associated with BMSC or HOB niches *in vivo* are more resistant to chemotherapy-induced apoptosis[11,18].

Based on previous works we sought to determine whether B- lineage acute lymphoblastic leukemia (ALL) cells, which share many common characteristics with their healthy pre- and pro-B cell counterparts, would localize to distinct compartments of BMSC or HOB co-culture resulting in distinct subpopulations for investigation of therapeutic resistance. We demonstrate that ALL cells recovered from the PD population of co-culture are phenotypically distinct, and exhibit many characteristics of refractory disease described *in vivo*. PD derived tumor cells are resistant to therapy with survival that approximates tumor cells that have not been exposed to cytotoxic agents. When compared to the other subpopulations recovered from the same co-culture, PD leukemic cells, in addition to their marked survival during chemotherapy exposure, were characterized by increased quiescence and elevated glycolytic activity. Our observations suggest that a biologically relevant model of minimal residual disease can be utilized *in vitro* that benefits from the inclusion of relevant human derived BMM constituents and targeted evaluation of the most resistant component of ALL. The PD leukemic cells in this model lend themselves to more rigorous drug screening than can be achieved when total leukemic populations are evaluated. Importantly, this novel approach of focus on the PD tumor cells may also more efficiently inform pre-clinical design to investigate MRD and relapse, with specific consideration of resistant subpopulations supported by the BMM.

Materials and Methods

Cell lines and culture conditions

Bcr;Abl (Ph+) lymphoblastic cell lines Tom-1 (DSMZ-ACC 578), Nalm-27 (Fujisaki Cancer Center), Nalm-30 (Fujisaki Cancer Center), and Sup-B15 (ATCC-CRL-1929) and (Ph-) REH (ATCC-CRL-8286) and Nalm-6 (ATCC-CRL-1567) were utilized. De-identified primary bone marrow stromal cells (BMSC) were provided by the Mary Babb Randolph Cancer Center (MBRCC) Biospecimen Processing Core and the West Virginia University Department of Pathology Tissue Bank. BMSC cultures were established as previously described[19]. Human osteoblasts (HOB) were purchased (PromoCell, Heidelberg, Germany), and cultured according to the supplier's recommendations. Co-cultures were established by seeding leukemic cells onto 80-90% confluent BMSC or HOB monolayers. Cultures were fed every 4 days and tumor cells collected for inclusion in experiments with remaining leukemic cells moved to new primary BMSC or HOB adherent layers every 12 days. Cultures were maintained in 5% O₂ to model normal bone marrow oxygen tension,

reported to range from 1-7% [20–22]. Suspended (S) leukemic cells floating freely in the media were removed by gentle pipetting. Phase bright (PB) tumor cells, that were loosely adherent to the top of BMSC or HOB, were harvested by vigorous pipetting. Phase dim (PD) leukemic cells that were buried firmly beneath adherent BMSC or HOB were recovered by trypsinization of the adherent layer and PD tumor. The S, PB, and PD tumor populations were separated from BMSC/HOB by size exclusion with G10 Sephadex (Sigma, St Louis, MO, USA) column separation [23,24].

Microscopy

Phase contrast images were acquired using a Leica DMIL LED microscope and processed by Leica application suite version 4.0 software (Buffalo Grove, IL, USA). Confocal images were acquired using an upright LSM 510 Zeiss microscope and processed using Zen2009 software (Thornwood, NY, USA). Fluorescence intensity for image acquisition was only altered when fluorescence intensities were not compared between samples.

Subpopulation tracking

The three ALL subpopulations were isolated from co-culture as described above. Each subpopulation (S, PB and PD) was individually stained with CellTracker™ Green, CellTracker™ Violet, or CellTracker™ Deep Red (Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. An equal number of cells from each population was combined and cultured on coverslips with confluent BMSC or HOB for 1, 4 and 48 hours. Coverslips were extensively washed with PBS to remove S and PB subpopulations, fixed with 4% paraformaldehyde, and stained with phalloidin-TRITC (Sigma). Slides were mounted with Prolong® Gold anti-fade (Life Technologies), and evaluated by confocal microscopy.

Chemotherapeutic agents

Cytarabine (Ara-C) (Selleckchem, Houston, TX, USA; Cat # S1648), Methotrexate (MTX) (Selleckchem, Cat # S1210) and Vincristine (VCR) (Selleckchem, Cat # S1241) were stored per manufacturer recommendations, and diluted in base media immediately prior to use. Experimental concentrations of Ara-C [1 μ M], MTX [50 μ M], or VCR [25 μ M] were used to approximate clinically relevant doses in ALL [25–29].

Evaluation of leukemic cell viability

ALL cells were cultured in media alone, or co-cultured with BMSC or HOB for 4 days to establish S, PB, and PD tumor populations. At day 4, cultures were provided fresh media and exposed to Ara-C, MTX, or VCR for 4 days. Cells treated with Ara-C were exposed to a second dose at 48 hours. Viability was evaluated by trypan blue exclusion in triplicate samples.

Annexin V/PI staining

Cell culture and chemotherapy exposure were completed as described above. Following drug exposure ALL cells were stained using an Annexin V (FITC)/propidium iodide (PI) apoptosis detection kit (Trevigen, Gaithersburg, MD, USA; Cat # 4830-01-K) according to

the recommended protocol of the manufacturer. Collection and analysis were performed in triplicate using the LSRFortessa (Becton Dickinson, San Jose, CA, USA).

Co-culture of tumor cells with non-bone marrow derived adherent cells

ALL cells were cultured in media alone, or co-cultured with sheep choroid plexus epithelial cells (SCP) (ATCC CRL-1700), 3T3 mouse embryonic fibroblasts (3T3) (ATCC-CRL-1658), mouse embryonic fibroblasts (MEF) (ATCC-CRL-2991), human embryonic kidney cells (293T) (ATCC-CRL-3216), or HT-1080 human fibrosarcoma derived cells (HT) (ATCC-CCL-121). Leukemic cells were cultured with adherent layers for 4 days to establish S, PB, and PD tumor populations comparable to those established with human BMSC/HOB. At day 4, cultures were exposed to Ara-C and evaluated for viability by trypan blue exclusion in triplicate as described above.

***In vitro* relapse model**

ALL cells were grown in co-culture with BMSC or HOB for 4 days. At day 4, cultures were provided fresh media and exposed to Ara-C for 72 hours. Following Ara-C exposure, S and PB ALL cell subpopulations were harvested as previously described and viability enumerated by trypan blue exclusion. Co-cultures, in which PD tumor cells remained buried beneath adherent BMSC/HOB, were rinsed to remove residual Ara-C and subsequently repopulation was monitored. ALL cells that comprised the regenerated S and PB fractions were enumerated at 5 day intervals at which time fresh media was provided. Cultures were maintained until tumor burden compromised BMSC or HOB monolayers.

Western blot analysis

Specific targets of glycolytic regulation were detected by rabbit mAb Hexokinase 1 (Cell Signaling Technologies, Danvers, MA, USA; C35C4; Cat # 2024) and rabbit mAb Hexokinase 2 (C64G5; Cat # 2867). Following incubation with horseradish peroxidase–conjugated secondary antibodies, signal was visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Densitometry was quantified by ImageJ with signal normalized to GAPDH.

Ki-67 staining

ALL subpopulations were either cytopun following G10 Sephadex purification following co-culture or analysis was completed on ALL cells during co-culture with BMSC or HOB grown on coverslips. Cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100 (Sigma) in PBS, and incubated with rabbit anti-Ki-67 followed by Alexa 488 anti-rabbit. Phalloidin-TRITC was used to visualize the actin cytoskeleton. Cells were washed with PBS and mounted to glass slides (coverslip staining) or coverslips (cytopins) with Prolong® Gold anti-fade/DAPI. Images were acquired by confocal microscopy and a minimum of 50 cells were counted to quantitate percent positive Ki-67 cells in triplicate.

Cell cycle analysis

G₀ accumulation of ALL cells was investigated by evaluation of the DNA/RNA content quantitated by Hoechst33342/Pyronin Y (H/PY) (Sigma) double staining as previously described.[30] To evaluate the overall cell cycle profile of ALL cells, leukemic cells were collected from media or the PD population of co-culture after 12 days. ALL cells were fixed in 70% ethanol, treated with RNase (Sigma), and stained with PI for DNA analysis. All samples were performed in triplicate and processed on a FACSFortessia flow cytometer, and analyzed by FCS Express 4 software.

Oxygen consumption and Extracellular Acidification analysis

Leukemic cells (800,000 cells/well) were collected from media alone or isolated from BMSC/HOB co-culture and incubated in an XF⁹⁶ cell culture microplate coated with Cell-Tak (BD Biosciences). The XF⁹⁶ Analyzer with XF Assay Media containing sodium pyruvate and glucose was used to determine Oxygen Consumption Rate [OCR] measurements, or with XF Base Media with L-glutamine to determine Extracellular Acidification Rate [ECAR] measurements. The XF plate was calibrated overnight with XF Calibrant at 37°C. On the day of the measurements, tumor cells were plated directly in XF media, and basal measurements (OCR or ECAR) were collected at three time points. All analysis was performed using XFe 96 Analyzer (Seahorse Bioscience, North Billerica, MA, USA)

Laser Ablation Electrospray Ionization (LAESI) and LC-MS/MS

Leukemic cells cultured in medium alone or harvested from the PD population of BMSC or HOB co-culture were analyzed by LAESI-MS for metabolic profiling. Quantitative proteomic LC-MS/MS analyses were performed on the following cell treatments: leukemic cells grown in media alone and co-culture with BMSC, BMSC/HOB, and HOB (Protea Biosciences, Morgantown, WV, USA). Detailed methodology and statistical analyses are included in Supplementary Materials and Methods.

Statistical analysis

Results are expressed as mean $\pm$ SEM. An unpaired t-test was performed to analyze results of cell viability data, H/PY, and KI67 quantitation (Graph Pad Software, La Jolla, CA, USA). Results of basal ECAR and OCR rates were analyzed by One-way ANOVA with Dunnett's post hoc (Sigma Plot, San Jose, CA, USA). A p value of < 0.05 was considered statistically significant as detailed in figure legends. Error bars throughout figures are represent standard error (SEM).

Results

ALL cells in co-culture with BMSC or HOB form three distinct spatial compartments

A panel of ALL cell lines formed three spatially and phenotypically distinct populations during co-culture with BMSC (Figure 1) or HOB (DNS). ALL cells were separated into suspended tumor (S), which are not in physical contact with BMSC/HOB, phase bright (PB) that are loosely adherent to BMSC/HOB and phase dim (PD) subpopulations which bury

beneath the BMSC or HOB monolayer (Figure 1). Each individual subpopulation reestablishes the PD subpopulation when collected and placed into subsequent co-cultures. When individual ALL subpopulations (S, PB, and PD) were removed from BMSC or HOB co-culture, stained with distinct dyes and re-plated for 1-48h of interaction with BMM, no unique affinity was observed within any group of ALL cells to repopulate the PD compartment (Figure 2).

ALL cells located in the PD population of BMSC or HOB co-culture demonstrate pronounced resistance to chemotherapy-induced death

PD ALL cells in BMSC/HOB co-culture have pronounced protection from chemotherapy-induced death when compared to media alone, S, or PB tumor cells following exposure to Ara-C, methotrexate (MTX), or vincristine (VCR) (Figure 3A-B and DNS). ALL cells readily establish three subpopulations (S, PB and PD) when co-cultured with several types of adherent layers established from sites other than the bone marrow, however, the PD population is not protected from Ara-C induced cell death (Figure 3C). Furthermore, we determined if surviving PD tumor cells from human BMSC or HOB co-culture could reconstitute the culture upon conclusion of chemotherapy exposure to simulate a purposefully simplified “*in vitro* relapse”. Following a quiescent period of approximately 15 to 20 days, ALL cells from the chemotherapy resistant PD population migrated from beneath the stromal layers and initiated proliferation to re-populate the cultures (Figure 3D).

ALL cells influenced by BMM have altered cell cycle distribution and quiescence

PD ALL cells have an increased G₀/G₁ fraction compared to media alone ALL cells (Figure 4A). The G₀/G₁ combined fraction was further investigated to determine the sub-set of PD ALL cells that are specifically in the G₀ (quiescent) phase by H/PY double staining. Corresponding to the increase in G₀/G₁, PD cells have an increase in the percentage of cells in G₀ compared to ALL cells in media alone (Figure 4B). Ki-67 staining was completed for analysis of the PD ALL population (Figure 4C) with PD ALL cells consistently having diminished Ki-67 staining when compared to media controls (Figure 4C-D). In addition to cell cycle analysis and Ki-67 expression alterations, global proteomic analysis was performed. As shown in Figure 5, proteins involved in cell cycle progression and DNA accessibility were altered in the leukemic cells influenced by the BMM. For a select group of histone-related proteins, alterations were seen in SUP-B15 ALL cells co-cultured with BMSC versus those co-cultured with HOB.

BMSC/HOB cues alter ALL metabolic profiles

PD ALL cells have an increased level of glycolytic activity reflected by increased extracellular acidification rate (ECAR) when compared to ALL cells in media alone (Figure 6A). In agreement with increased basal glycolytic activity, PD ALL cells also have increased Hexokinase 1 and 2 protein levels (Figure 6B). While glycolytic activity was increased in the PD population, basal levels of oxidative phosphorylation are significantly diminished in leukemic cells in co-culture (Figure 6C). Metabolomic analysis was completed to compare global changes between leukemic cells in the presence (PD) or

absence (media alone) of microenvironment cues with clustering of m/z according to expression level reflecting distinct metabolic profiles (Figure 6D).

Discussion

In this study, using an extension of 2D co-culture techniques we have demonstrated that a panel of ALL cell lines when in co-culture with BMSC or HOB form three distinct spatial compartments: S, PB, and PD. Of these, the PD population is the most resistant and quiescent subpopulation generated by BMM interactions *in vitro*, and provides a valuable tool that can be applied for studying aggressive, resistant leukemia for targeted intervention. To evaluate whether the three individual groups of ALL cells had specific affinity for the location from which they were isolated, cell tracking experiments were completed. Leukemic cells in our model behave stochastically, and did not preferentially reconstitute the location from which they were isolated (Figure 2). S, PB or PD derived ALL cells readily buried under BMSC or HOB (“became PD”). This observation suggests a very dynamic phenotype, driven by the specific cues in distinct locations, versus a sustained phenotypic alteration or ALL cell “memory”.

Our laboratory has previously shown that in general BMSC provide chemotherapy protection to leukemic cells via soluble factors[32,33] and adhesion interactions in standard 2D co-cultures[15,34–36]. When the attention was directed to the PD subpopulation, they were shown to be the most resistant to chemotherapy exposure. Protection was specific to interactions with BMM-derived BMSC, HOB or NSG derived BMSC (Figure 3A-C, DNS). The value of our model approach is the observation that, with inclusion of just one niche component, either BMSC or HOB, a distinct group of tumor cells can be isolated that have altered cell cycle profiles, metabolic signatures and cytotoxic agent response. Future work can expand upon this model, increasing its complexity through methodical addition of other key bone marrow microenvironment components, such as endothelial cells[36], to better recapitulate the resistant leukemic niche *in vitro* to answer specific, clinically relevant questions with emphasis on interruption of signals that influence ALL cell quiescence, metabolic activity, and apoptosis.

Tumor cells that are in a quiescent state have been described by other groups to be drug resistant[37]. We observed that PD cells accumulate in the G₀/G₁ phase of cell cycle with a reduction in S phase (Figure 4A). Coincident with induction of quiescence, PD cells had increased p27 suggesting this may be one mechanism by which the cells are arrested in a G₀ state (DNS; figure 4B). The PD population does not demonstrate 100% accumulation in G₀ indicating that cell cycle interruption does not explain the entire escape from S phase specific drug-induced apoptosis. Even within the purified PD subpopulation multiple pathways are likely influenced by niche cells that converge on blunting apoptosis. PD ALL cells also had a reduced proliferation index measured by Ki-67 staining (Figure 4C-D), again consistent with the assertion that the BMM alters ALL proliferation, which may be one contributing factor to increased chemotherapy resistance (Figures 3-4). Future studies will be important to evaluate a broader range of drugs in this model, including those that are not S phase specific, to expand our understanding of the resistant subpopulation.

Diverse protein pathways were altered in leukemic cells during co-culture with BMM cells evaluated by MS/MS analysis (Figure 5). Key pathways with significant alteration in ALL cells were those associated with cell cycle progression and DNA accessibility. Interestingly, differences were not only seen in ALL cells from BMM niche co-culture compared to media alone, but also in histone related protein expression between ALL cells influenced by BMSC compared to HOB. Although both cell types are derived from the bone marrow, it is not surprising that they influence ALL gene expression differently based the uniqueness of the endosteal and perivascular niches[38,39]. This reflects the complexity of the marrow niche and compartmentalization of function that is critical for regulated development of healthy hematopoietic cells through controlled differentiation, proliferation, survival and eventual egress to the periphery.

We further characterized the metabolomic profiles of the PD subpopulation compared to tumor cells grown in media alone utilizing LAESI-MS. The metabolic signatures of leukemic cells provided microenvironment cues were notably distinct from ALL cells deprived of these signals (Figure 6). This global evaluation was not intended to identify specific targets but rather to determine if overall metabolite patterns were shifted (Figure 6D). The cellular metabolism of this subpopulation of leukemic cells has been altered by the bone marrow microenvironment interactions, resulting in quiescent cells with high glycolytic potential (Figure 6A-B). This has been previously observed in quiescent fibroblasts[40], and the notion that the PD cells are “primed” for proliferation when they are released from quiescent constraints is a characteristic previously reported in activated dormant T cells[41].

A particular concern that remains in ALL is the aggressive treatment strategies required to treat relapse in children with the marked potential of late effects and secondary malignancies. Against this backdrop, it is essential to expand the knowledge we obtain *in vitro* for efficient translation to pre-clinical modeling, subsequent novel drug trials, and generation of new treatment paradigms for patients. Clearly limitations are significant in modeling a complex and dynamic disease such as ALL *in vitro*. The unique leukemic co-culture model described with an emphasis on a functionally distinct, quiescent and chemoresistant PD subpopulation provides a valuable tool for future analysis. Further dissection of the molecular pathways regulated by the BMM and the “sanctuary” that is created for leukemic cells to generate refractory disease still needs to be extensively studied in order to achieve a higher percentage of disease free survival in leukemia patients.

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- *In vitro* model of bone marrow microenvironment supports a resistant ALL subpopulation
- *In vitro* bone marrow microenvironment establishes 3 distinct subpopulations of ALL
- Phase Dim ALL cell subpopulation are characterized by an increase in quiescence, chemotherapy protection and altered metabolic prolife

Figure 1.

Human acute lymphoblastic leukemia cells (ALL) occupy three functionally distinct spatial compartments during co-culture with BMSC. The left panels show suspended ALL tumor cells from representative cell lines (REH, Nalm-27 or Tom-1) grown in media alone. Right panels demonstrate co-culture dynamics with leukemic cells forming three functionally distinct populations in relation to the BMSC monolayer. Cells were co-cultured for 48 to 72h prior to acquiring images. The three ALL populations include suspended (S) cells which refract light, appear bright and are freely floating in culture media; phase bright (PB) tumor cells that are loosely attached to the surface of the adherent BMSC monolayer; and phase dim (PD) tumor cells which are beneath the BMSC monolayer and appear as phase dim cells upon light microscopy. Arrows highlight the PD subpopulation of ALL cells in co-culture with BMSC, which are enlarged in the inset for more detailed visualization. Scale bar= 10 μ m.

Figure 2.

Each subpopulation of Nalm-27 ALL cells re-established the PD fraction when provided BMM niche cells. The S, PB and PD ALL subpopulations were collected from co-culture and individually stained with blue, far red and green fluorophores for visualization, respectively. Each subpopulation associated with BMSC (top panel) and HOB (bottom panel) observed after 48h. Co-cultures were vigorously rinsed to remove S and PB tumor cells to visualize only PD tumor cells shown in the representative images. Actin was visualized by Phalloidin-TRITC staining. Levels including gamma were adjusted and a median filter was applied for image clarity with no comparison of intensity. Scale bar= 10 μ m.

Figure 3.

PD leukemic cells have pronounced resistance to chemotherapy induced death. **(A)** Leukemic cells recovered from the PD population of an HOB co-culture have significantly increased viability following exposure to Ara-C [1 μ M], MTX [50 μ M], or VCR [25 μ M] determined by trypan blue exclusion or **(B)** Annexin V-FITC/PI staining when compared to matched controls that are exposed to cytotoxic agents in media alone. **(C)** In contrast to human BMSC or HOB co-culture, leukemic cells recovered from the PD population of non-bone marrow derived adherent layers do not have increased viability compared to untreated controls during exposure to Ara-C. **(D)** PD leukemic cells undergo a quiescent recovery period following Ara-C exposure, followed by an aggressive period of proliferation to repopulate the S and PB populations. (*) denotes $p < 0.05$, unpaired t-test when compared to media alone or untreated controls.

Figure 4.

BMM cells influence the PD subpopulation cell cycle profile and increase quiescence. **(A)** PD cells have a higher percentage of cells in the G₀/G₁ phase and a reduction in S phase compared to media alone leukemic cells as measured by PI staining. **(B)** H/PY staining revealed the PD cells have a higher percentage of cells in G₀ when compared to tumor cells cultured in media alone. **(C)** Assessing Ki-67 (green) expression by confocal microscopy, the PD cells had a reduction in Ki-67 positive cells. TRITC staining: red, DNA staining: blue, Ki-67: green. Scale bar equal= 10 μ m. (*) denotes $p < 0.05$, unpaired t-test when compared to media alone.

Figure 5. BMM alters global protein expression in ALL cells. The heat map shown represents differentially regulated proteins comparing Sup-B15 ALL cells co-cultured with BMSC or HOB to those grown in media alone. GAPDH was used as a control to normalize expression in all groups. A p-value <0.05 for comparisons of co-culture relative to media alone was required for designation of a difference in protein abundance.

Figure 6.

PD cells isolated from bone marrow niche co-culture have a distinct metabolic phenotype when compared to ALL cells in the absence of microenvironment signals. **(A)** REH cells recovered from the PD population have increased basal glycolytic rates compared to tumor in media alone or their S and PB counterparts from the same BMSC or HOB co-culture as indicated by the increase in extracellular acidification rate (ECAR). **(B)** Western blot analyses indicated increased Hexokinase 1 and Hexokinase 2 protein expression in PD ALL cells compared to tumor cells maintained in media alone. **(C)** REH cells in co-culture with BMSC or HOB have decreased basal oxygen consumption compared to media alone controls as reflected by diminished oxygen consumption rate (OCR). **(D)** Laser ablation electrospray ionization mass spectrometry (LAESI-MS) metabolomic analysis of Nalm-27 cells cultured in media alone or recovered from the PD population of BMSC or HOB co-culture showed global changes of metabolites between ALL cells populations. Heat map represents differentially expressed metabolite ions (m/z values), p-value < 0.01. (*) denotes $p < 0.05$ by One-way ANOVA with Dunnett's post hoc when compared to media alone.