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Niyati Upadhyay April 9, 2019

A Study of the Impact of the Obese Microenvironment on Human Leukemia Cells

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Abstract

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Cancer research has seen a dramatic increase in the number of deaths relating to obesity. Adipocytes have been thought to be drivers of cancers such as leukemia, although the exact mechanism by which they confer resistance to chemotherapy drugs and promote tumor formation is unknown. A previous study by Sheng *et. al* indicates that adipocytes secrete some sort of protective factor that prevents apoptosis in acute lymphoblastic leukemia (ALL) cells. The primary goal of this project is to uncover the various pathways by which adipocytes confer resistance and promote leukemogenesis in order to improve upon treatment for both obese and lean patients. This was done by looking at cell death, cell proliferation, and gene expression profiles of various T-ALL and B-ALL cell lines. While the data provided ample evidence for how adipocytes impact the survival and proliferation of leukemia cells, future studies must be done to quantify gene expression specific to certain pathways. Targeting such pathways will allow for more effective and localized chemotherapy drugs and treatments.

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1) Introduction

1.1) Epidemiology of Leukemia

Leukemia impacts about 40,000 adults per year and about 2,000 children per year, with these numbers continuing to rise[1]. Roughly 33% of all cancers diagnosed between birth and 14 years of age are leukemias, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML) subtypes[1]. Of these, ALL is the most common[2]. The overall cure rates for pediatric ALL is over 90%; however, survival in patients with minimal residual disease (MRD), the number of leukemia cell that remain after treatment or at diagnosis, or those who relapse have an overall 5-year survival rate of less than 30%[1]. Furthermore, patients who fall into high risk categories including the elderly and those who are overweight or obese, have an overall 5-year survival rate of less than 70% for certain subtypes of leukemia[1].

It is estimated that between 14%-20% of all deaths relating to cancer come from the detrimental effects of obesity[3]. Childhood obesity also reduces survival outcomes in children with ALL[4]. This dismal prognosis can be partially attributed to a significant increase in relapse rates observed in obese children with ALL. The same observations are true for obese adults with ALL, where there is nearly a 50% increase in the likelihood of relapse rates compared to lean patients. In addition to assaying overall survival (OS) in patients with leukemia, minimal residual disease (MRD) is a commonly used prognostic marker for event-free survival (EFS) post-treatment. Using this metric, EFS was also observed to be significantly lower in overweight and obese pediatric patients with ALL[4]. Based on the mounting evidence that obesity promotes ALL disease progression and significantly reduces survival in pediatric and adult patients, it is imperative that we begin to understand how adiposity impacts the function of leukemia cells in

order to developed more effective therapeutic strategies for this high-risk population. Given that obese patients with colon, breast, and prostate cancer also exhibit poorer survival outcomes, a more thorough understanding of the relationship between obesity and carcinogenesis is needed to improve treatment outcomes in this high-risk demographic[5].

1.2) Adipocytes as Cancer Drivers

Adipocytes accumulate with weight gain and have recently been shown to alter the pharmacokinetics and pharmacodynamics of chemotherapies *in vitro* and in murine models. Indeed, adipocytes metabolize drugs to their inactive forms and release pro-inflammatory cytokines and chemokines that can alter the cellular microenvironment and impact the function of non-malignant and malignant cells. Based on these properties and their impact on disease progression and chemotherapeutic efficacy, adipocytes are thought to be drivers of many solid tumors.

The role of adipocytes in hematological malignancies is certainly less clear. Interestingly, it has been shown that adipocytes accumulate in the bone marrow during chemotherapy[6] treatment and this altered bone marrow microenvironment is thought to promote chemoresistance. Gene expression analysis of adipose tissue isolated from normal donors and patients with breast cancer, colorectal cancer, non-Hodgkin's lymphoma and myeloma show an alarming genetic overlap between pathways activated in adipocytes from obese patients and those with the aforementioned cancers[7]. Some of the pathways linked to obesity and the prevention of apoptosis (which induces cell proliferation) include those involving adipokines, sex hormones, and insulin-like growth factors (IGFs)[7]. Some genes associated with obesity and

carcinogenesis include IGF1, SSTR5, IGFBP3, and IGFALS[7]. The gene expression changes, however, on cellular metabolism and disease progression remains to be determined.

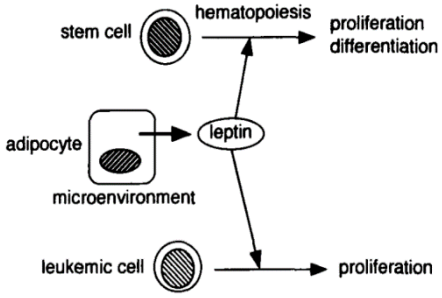
1.3) Adipocytes and Cellular Metabolism

Metabolism regulates cellular proliferation, survival, and other biological processes both at homeostasis and under conditions of stress. Adipocytes alleviate oxidative stress in ALL cells exposed to the chemotherapeutic drug daunorubicin which is induced in part by adipocyte-secreted factors[8]. Cell-to-cell contact is not needed for resistance to daunorubicin, determined by allowing media to freely diffuse between ALL cells in a TransWells set up with a polycarbonate membrane[8]. Indeed, ALL cells exposed to adipocyte-conditioned media (ACM) upregulate antioxidant pathways highlight by the strong induction of Nrf2 and downstream target genes[8]. The ability to mitigate chemotherapy-induced oxidative stress resulted in less cytotoxic in ALL cells after exposure to daunorubicin.

The adipocyte-secreted, chemoprotective factors are currently undefined; however, we are slowly beginning to understand how the adipocyte secretome confers chemoresistance to ALL cells. Since ALL cells require asparagine (ASN) and glutamine (GLN) for proliferation, quantifying ASNase activity in ALL cells and ASN and GLN levels in adipose-rich microenvironments are useful biomarkers that may predict chemotherapy efficacy[9]. ASNase is an enzyme that breaks asparagine and glutamine down into aspartic acid and glutamic acid (which is regulated by the concentrations of each amino acid). Obesity reverses the depletion of glutamine levels in the body because adipocytes produce copious amounts of asparagine and glutamine[9]. The elevated levels of these amino acids decreased apoptosis and increased proliferation in ALL cells in the bone marrow of mice[9]. Together, these results highlight how

elevated levels of GLN and ASN in adipose-rich microenvironments promote ALL disease progression and chemoresistance to front-line chemotherapies[10].

Adipocytes are also involved in producing various cytokines that may alter chemosensitivity in ALL cells. Adipocytes produce leptin, a protein known to induce proliferation and differentiation of hematopoietic cells (**Scheme 1**). Leukemic cells contain cytokine receptors for interleukin-3 (IL-3), interleukin-6 (IL-6), and leptin, suggesting that these cytokines may impact the function of leukemia cells and are used as inflammatory markers[11-12]. Indeed, leptin has been shown to increase the number of AML progenitor cells and their proliferative capacity[13]. It also prevents apoptosis in leukemia cells in cytokine-deprived conditions[13].



Scheme 1. Adipocyte-produced leptin promotes hematopoiesis and leukemogenesis

Interleukin-6 also induces B-cell differentiation and proliferation[14]. Obesity is associated with local (bone marrow) and systemic increases in IL-6; therefore, elevated levels of IL-6 in obese microenvironments may promote B-cell leukemogenesis.

In addition to leptin and IL-6, Chen and colleagues[12] found that IGF-1 increases the levels of other cytokines (such as IL-6, IL-17, and TNF-alpha) in the microenvironment which induce proliferation and cancer initiation via the activation of the phosphoinositide 3-

kinase/protein kinase B pathway (PI3K/Akt), mitogen-activated protein kinase (MAPK) pathway and signal transducer and activator of transcription 3 (STAT3) pathways[12]. Leptin also stimulates these pathways by activating targets such as RhoA, Cdc42, and Rac1 which are associated with enhanced cancer cell proliferation and aggression. These studies highlight how obesity-induced changes in the microenvironment promote leukemogenesis and reduce therapeutic efficacy. Importantly, these studies demonstrate a need to development novel strategies that target the adipose-rich microenvironment to improve overall survival in overweight and obese patients with ALL.

2) Results and Discussion

2.1) Proteomics

Mass spectrometry analysis of unconditioned media (DMEM), stromal cell-conditioned media (SCM), and adipocyte-conditioned media (ACM) was used to identify differences in proteins secreted by stromal cells (which do not confer chemoresistance) and adipocytes (which induce chemoresistance). Our initial experiment was performed without trypsinization, which allows for the detection of smaller proteins and peptides, and subsequent studies will be performed with trypsinization to identify larger proteins that are secreted by adipocytes. Our results revealed striking differences in the spectrum of small proteins and peptides secreted by adipocytes relative to stromal cells (**Figure 1**).

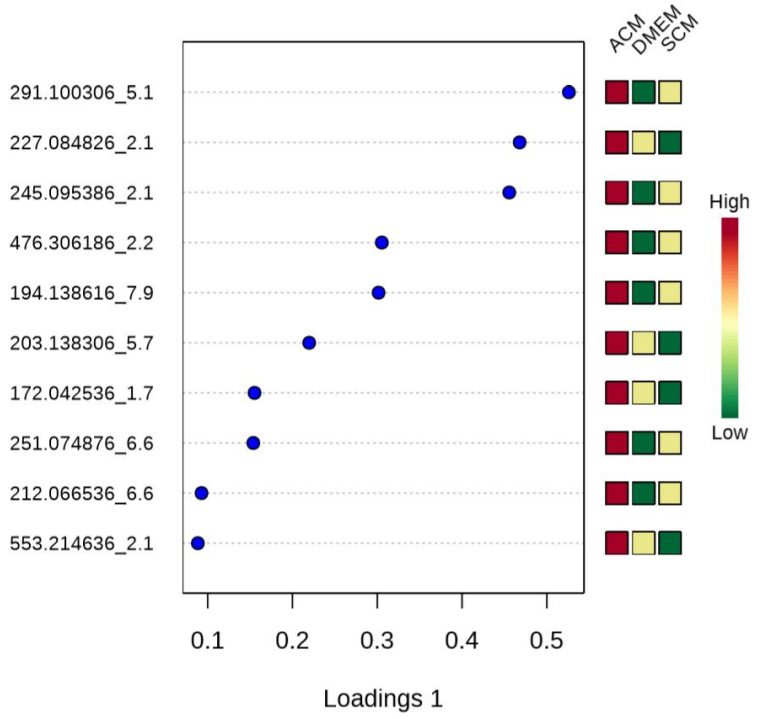


Figure 1. sPLS-DA plot of the top 10 differentially expressed proteins present upon exposure to ACM, SCM, and DMEM (control). The adipocyte secretome is drastically different than factors secreted by bone marrow cells.

2.2) Cell Cycle Data and EdU Analysis

Given the striking difference between soluble factors secreted by bone marrow stromal cells and adipocytes, we next tested how SCM and ACM (as well as their respective secretomes) impacted the proliferation of human B-leukemia cells. Using EdU analysis, we found that ACM exposure promotes S to G2 transition in human B-ALL cells (KOPN8 and REH cells), albeit to differing extents (**Figure 2B and 2C**). We tested 9 human B-leukemia cell lines in these studies, and we found that ACM (but not SCM) exposure promoted cell cycle progression in every human B-ALL cell line tested (**data not shown**). While we see a promotion of cell cycle progression in all B-ALL cell lines, the largest differences are seen in the KOPN8 cells between S and G2. The EdU plot indicates a higher quantity of DNA found in the G2 mitotic phase compared to the S phase in KOPN8 cells (**Figure 2A**).

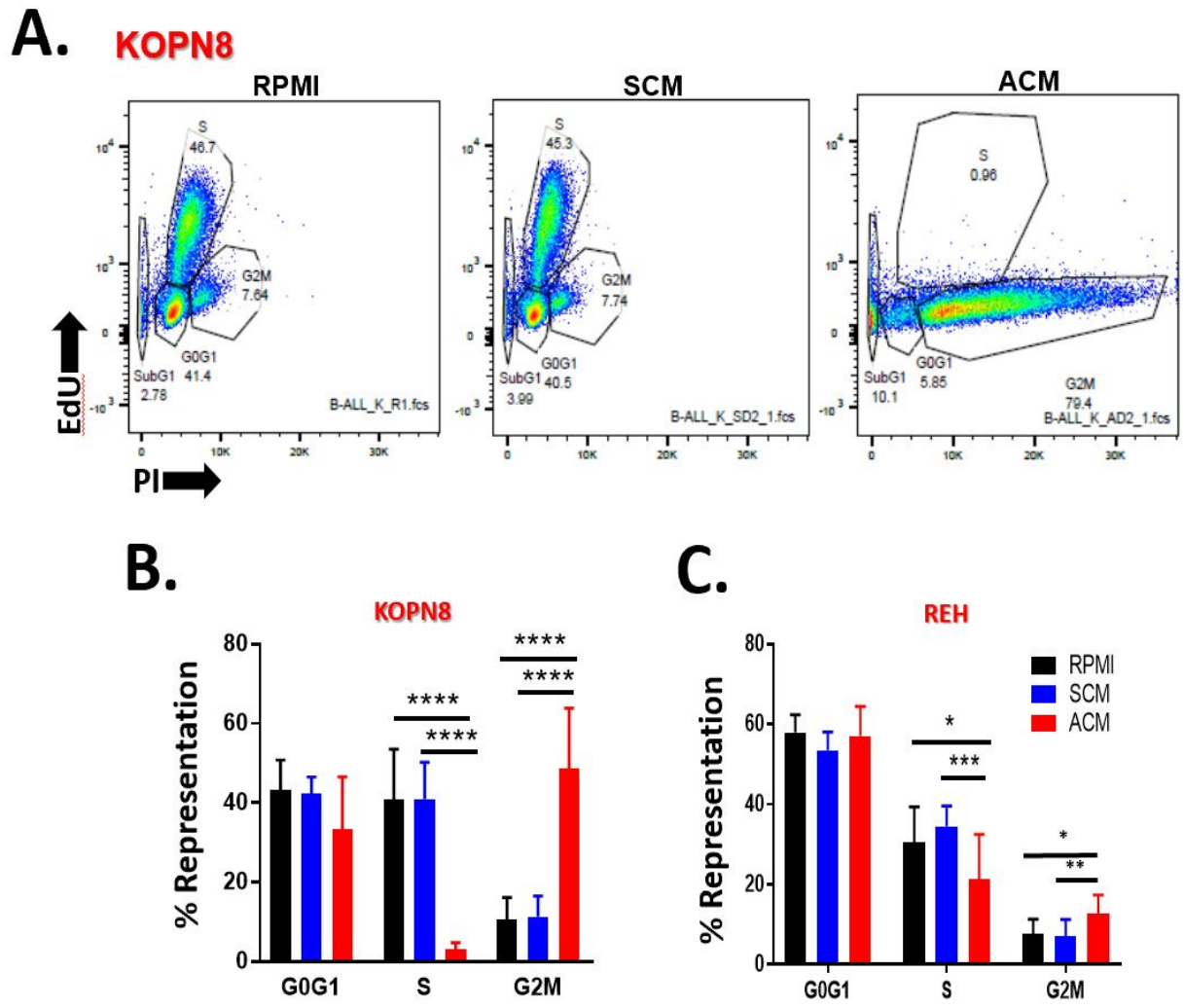


Figure 2: EdU data **(A)** of KOPN8 cells in RPMI, SCM, and ACM with the x-axis indicating DNA quantity and the y-axis indicating DNA intensity. Representation of KOPN8 **(B)** and REH **(C)** leukemia cells in various mitotic stages. (n=3, two-way ANOVA, *=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001).

2.3) Apoptosis

Given that ACM induced cell cycle progression in every human B-leukemia cell line tested, we next wanted to determine the impact of ACM on apoptosis alone and in response to chemotherapy exposure in 9 human B-leukemia cell lines. We found that ACM has differing effects on KOPN8 and REH cells. When KOPN8 cells were exposed to ACM, greater than 70% of the cells died; whereas, only 30% of REH cells died when exposed to ACM (a representative KOPN8 response is shown in **Figure 3A** and the combined data from multiple experiments for both cell lines are shown in **Figure 3B**).

To determine how ACM exposure impacted chemosensitivity in human B-ALL cells, leukemia cells were pretreated with ACM for 24 hours prior to exposure of the chemotherapeutic drug methotrexate (MTX). We decided to pretreat cells prior to chemotherapy exposure to mimic what would occur in obese patients receiving chemotherapy treatment. Pretreating 8 of 9 human B-ALL (including REH cells) resulted in the significant chemoresistance (combined REH results are shown in **Figure 3D**). This gives weight to the idea that there is a protective quality present in the secretome of adipocytes that may confer this resistance to cell death of REH cells. Overall, 88% of the human B-leukemia cell lines test exhibited significant reduction in MTX-induced apoptosis if they were exposed to adipocyte-secreted factors prior to chemotherapy exposure.

The only human B-leukemia cell line that remained responsive to MTX-induced cytotoxicity were KOPN8 cells (representative primary data are shown in **Figure 3C** and combined results from 4 experiments are shown in **Figure 3D**). This is because cell death has already reached a maximum from factors secreted by adipocytes that adding a drug does not have as great of an impact.

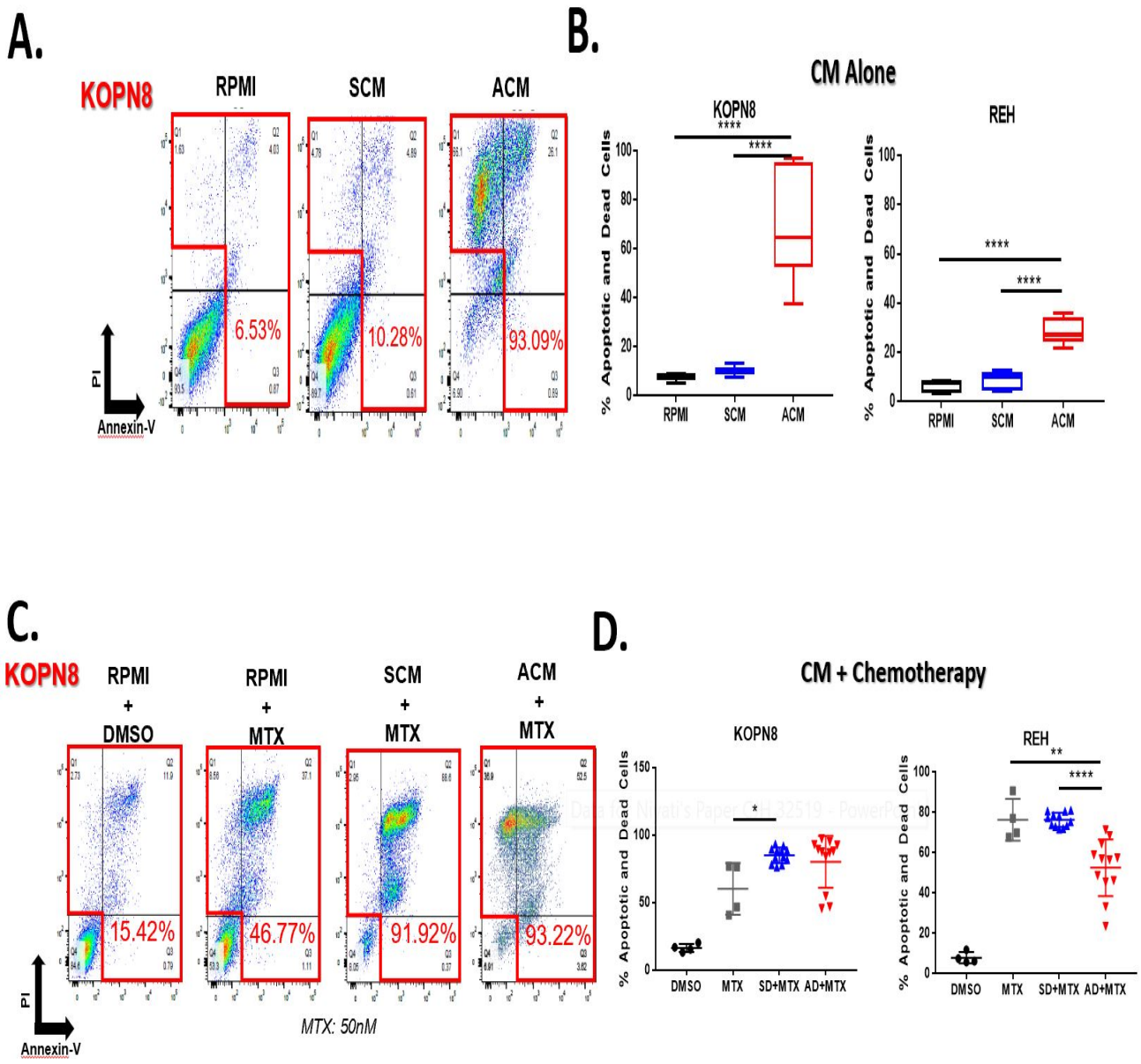


Figure 3. Annexin-V data of KOPN8 cell death in conditioned medias alone **(A)**, cell death and apoptosis levels in both KOPN8 and REH cell lines in conditioned media alone **(B)**, Annexin-V data of KOPN8 cell death in conditioned medias and methotrexate (MTX) **(C)**, cell death and apoptosis levels in both KOPN8 and REH cell lines in conditioned medias and MTX **(D)**.

*=p<0.05, **=p<0.01, ***=p<0.005, ****=p<0.0001

2.4) Changes in Gene Expression Profiles and RNA Sequencing

Given the significant induction of chemoresistance mediated by ACM, we decided to identify changes in gene expression profiles in human B-ALL that correlated with chemoresistance and chemosensitivity (using KOPN8 cells as controls for the chemosensitive response). To this end, we performed RNA-sequencing analysis on KOPN8 (ACM does not confer chemoresistance), REH (ACM confers chemoresistance), and RCH (ACM confers chemoresistance; **data not shown**) human B-ALL cells after exposure to conditioned media alone and in combination with MTX.

PCA analysis, which bins gene expression changes relative to profiles found in other experimental cell types using unbiased algorithms, revealed that ACM exposure alone induced significant gene expression changes in KOPN8 cells (**Figure 4A**). This global change in gene expression profiles correlates with the extensive apoptotic response observed in these cells in response to ACM exposure alone (**Figures 3A and 3B**). In contrast, REH cells did not exhibit extensive global gene expression changes when exposed to ACM alone (**Figure 4B**), which was also observed in RCH-AcV cells (**data not shown**). These observations suggest that cells with more genetically stable profiles respond to ACM exposure by not drastically altering their global programs but instead modulate specific pathways which promote chemoresistance.

This may be due to the nature of the KOPN8 cell line, as it is a quasi-B/T cell leukemia. T-ALLs are known to have genomic instability, causing cell death upon exposure to ACM due to the induction of proliferation. B-ALL cells perhaps confer more stability because they behave more like stem cells and are senescent. Because KOPN8 cells have T-cell characteristics, they might not be protected by ACM when exposed to drugs due to their instability. REH cell lines

have primarily B-cell characteristics and perhaps have a more stable genome, allowing them to be resistant to chemotherapy drugs in ACM.

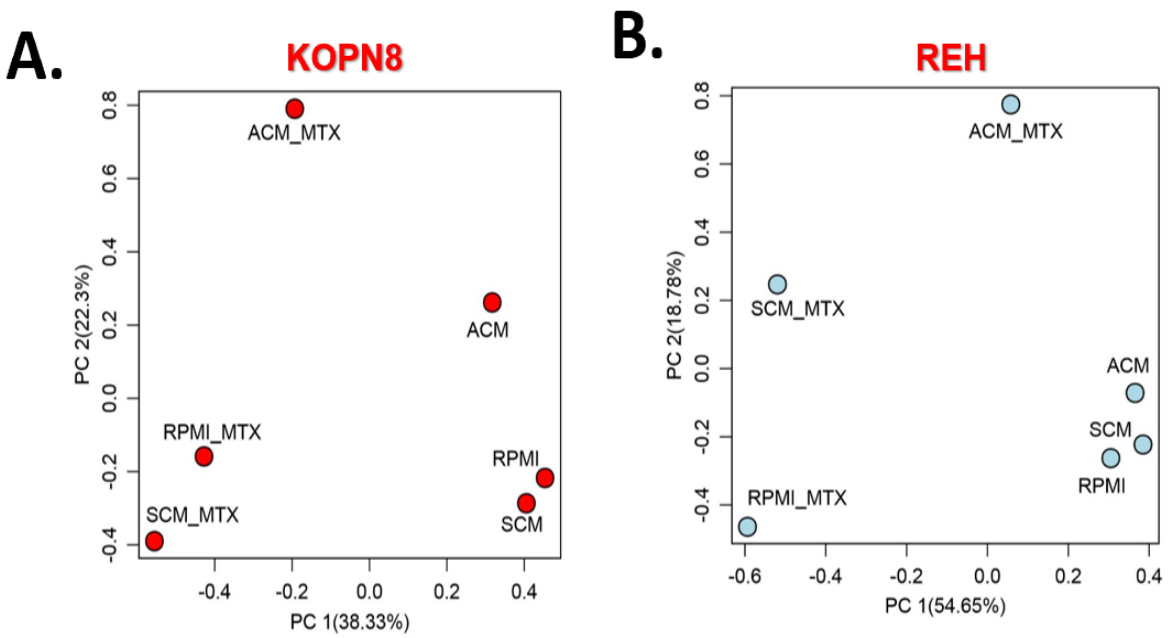


Figure 4. Principal Component Analysis plots for KOPN8 (**A**) and REH (**B**) cells conditioned in ACM, SCM, and RPMI both with and without MTX

In order to identify specific genes from our gene expression analysis that were involved in ACM-induced chemoresistance, we collaborated to perform bioinformatic analysis of our gene expression results. The significantly altered genes that increase and decrease in KOPN8 cells (chemosensitive) and REH cells (chemoresistant) after ACM exposure alone and in the presence of MTX are shown in **Tables 1-4**. From these analyses, interesting genes appeared to track with chemosensitivity. For example, the FOS gene is significantly upregulated in ACM exposed REH cells (**Table 2**). Increased expression of this gene promotes cell proliferation and survival in immune cells and may also increase cell cycle progression and survival in B-leukemia cells

exposed to adipocyte-secreted factors. Additionally, the PTEN tumor suppressor gene is also downregulated in ACM-exposed REH cells treated with MTX. Failure to induce PTEN activation in B-leukemia cells exposed to MTX could result in continued cell proliferation and survival, thus preventing the cytostatic and cytotoxic effect of this drug (**Table 4**). These pathways and others highlighted in the tables, could be future targets of chemotherapeutic drugs to overcome adipocyte-induced chemoresistance in B-leukemia cells.

Top Genes Increased in Chemosensitive Human B-ALL when treated with ACM	Top Genes Decreased in Chemosensitive Human B-ALL when Treated with ACM
ITCH (E3 Ubiquitin Ligase; increases protein turnover)	CALM2 (Calmodulin 2; regulates calcium activation)
RACGAP1 (Rac GTP-activating protein; promotes cellular proliferation)	TOM1L1 (Target of Myb1 Like 1; involved in membrane trafficking)
RHOA (Ras homolog gene family; member A- regulate actin cytoskeleton function and actomyosin contractility)	PTEN (Phosphatase and tensin homolog; tumor suppressor gene that halts cell cycle progression and promotes apoptosis)
EGR1 (Ether-a-go-go-Related Gene; codes for the alpha subunit of a potassium ion channel)	ITGB4 (Integrin, beta 4; involved in cell adhesion)
	GCLC (Gluamate-cysteine ligase catalytic subunit; involved in glutathione synthesis which buffers oxidative stress)

Table 1. Upregulated and downregulated genes present in **chemosensitive** B-ALL cells upon exposure to adipocyte conditioned media (ACM)

Top Genes Increased in Chemoresistant Human B-ALL when treated with ACM	Top Genes Decreased in Chemoresistant Human B-ALL when Treated with ACM
FOS (Forms AP1 when combined with c-Jun; promotes proliferation and survival of lymphoid cells)	SH3BGRL (SH3 domain-binding glutamic acid-rich-like protein; regulates redox activity as a oxidoreductase)
SMU1 (involved in pre-mRNA splicing as a component of the splicesome)	SEMA6A (Semaphorin 6A; plays an important role in cell-to-cell signaling. Promotes reorganization of the actin cytoskeleton and migration)
PTPRA (Receptor-type tyrosine-protein phosphatase alpha; dephosphorylates and activates Src family kinase which regulates integrin signaling, cell adhesion, and proliferation)	LRPAP1 (LDL Receptor Related Protein Associated Protein 1; facilitates the proper folding and localization of the LDL receptor by preventing binding of ligands)
EGR1 (Ether-a-go-go-Related Gene; codes for the alpha subunit of a potassium ion channel)	CKMT2 (Creatine Kinase S-type, mitochondrial; involved in charging ATP and shuttling it to the mitochondria for cellular usage)
TUBB4A (tubulin beta 4A class Iva; part of microtubules that aids in cellular movement)	

Table 2. Upregulated and downregulated genes present in **chemoresistant** B-ALL cells upon exposure to adipocyte conditioned media (ACM)

Top Genes Increased in Chemosensitive Human B-ALL when treated with ACM + MTX	Top Genes Decreased in Chemosensitive Human B-ALL when Treated with ACM + MTX
Not Reported	RAN (RAs-related Nuclear protein, a GTP-binding nuclear protein)
Not Reported	ADCY4 (Family of adenylate cyclases that catalyze formation of secondary messenger cyclic adenosine monophosphate (cAMP))
Not Reported	PLA2G4B (Hydrolyzes the sn-2 bond of phospholipids, releasing lysophospholipids and fatty acids)
Not Reported	AK7 (Phosphotransferase that catalyzes the phosphorylation of adenine)
Not Reported	RABEP1 (Recycling endosomes and endocytic membrane fusion)

Table 3. Upregulated and downregulated genes present in **chemosensitive** B-ALL cells upon exposure to adipocyte conditioned media (ACM) and methotrexate (MTX)

Top Genes Increased in Chemoresistant Human B-ALL when treated with ACM +MTX	Top Genes Decreased in Chemoresistant Human B-ALL when Treated with ACM + MTX
NFATC4 (Nuclear factor of activated T cells (NFAT), part of a DNA-binding transcription complex)	GADD45A (Growth Arrest And DNA Damage Inducible Alpha; increased during growth arrest situations)
HLA-E (Major Histocompatibility Complex, Class I, E; binds to membranes and a subset of peptides)	ZFP36L1 (Early response gene)
TSC1 (Tumor suppressor gene; encodes hamartin, a growth inhibitory protein)	PTEN (Tumor suppressor gene)
ADCY4 (Family of adenylate cyclases that catalyze formation of secondary messenger cyclic adenosine monophosphate (cAMP))	RHEB (Ras homolog; regulates growth and cell cycle progression)
RAD17 (Cell cycle checkpoint gene required for cell cycle arrest and DNA damage repair in response to DNA damage)	GGCT (Gamma-Glutamylcyclotransferase; plays a role in cell proliferation and potential cancer marker)
CHRNA5 (Cholinergic Receptor Nicotinic Alpha 5 Subunit; mediate fast signal transmission at synapses)	STRADB (Serine/threonine protein kinase family; regulates cell polarity and metabolism)
MYD88 (Signal transducer in the interleukin-1 pathway)	TMEM189 (A transmembrane protein when coded with ubiquitin-conjugating enzyme E2 variant 1)
	CHRNG (Cholinergic Receptor Nicotinic Gamma; transmembrane glycoprotein)
	GRIN3B (Creates an excitatory glycine receptor when combined with GRIN1)
	ARHGEF7 (Activates Rho proteins by using GTP; involved in cell migration and cell spreading)
	RAN (RAs-related Nuclear protein, a GTP-binding nuclear protein)

Table 4. Upregulated and downregulated genes present in **chemoresistant** B-ALL cells upon exposure to adipocyte conditioned media (ACM) and methotrexate (MTX)

3) Conclusions

Experiments and analyses were conducted to determine what role adipocytes play in chemotherapy resistance in various human B-ALL cell lines. It was determined that ACM causes more cell proliferation and death in KOPN8 cells compared to 8 additional human B-ALL cell lines, including REH cells. The differences in apoptosis and chemosensitivity to ACM exposure could potentially be attributed to the genomic stability inherent to the exposed leukemia cells. For example, KOPN8 cells express both B- and T-cell markers indicating that these cells may be experiencing "epigenetic confusion" unlike the other 8 human B-ALL cell lines that only express B-lineage markers.

A list of genes was compiled from the RNA-sequencing analysis to determine genes in human B-leukemia that are altered after exposure to adipocyte-secreted factors alone and in combination with MTX. Candidate genes that should be considered for targeting to overcome ACM-induced chemoresistance include FOS (given that this gene increases cellular proliferation and survival in immune cells) and the tumor suppressor PTEN (given that ACM exposure significantly decreased the expression of this gene in leukemia cells). Taken together, these findings show that adipocyte-secreted peptides and protein significantly alter gene expression in leukemia cells which correlate with increased cell cycle progression, and in most cases, the induction of chemoresistance.

4) Future Directions

Since reactive oxidative species are byproducts of metabolism, reactive oxidative species (ROS) levels can indicate if the cell is undergoing metabolic stress in various microenvironments. Experiments in progress are focused on determining if ACM exposure significantly alters ROS levels in leukemia cells. We will detect cytoplasmic ROS using CellRox Deep Red dye[15] and 2',7'-di-chlorofluorescein (DCF). The acetylated form of DCF, H_2DCFDA, is not fluorescent until oxidation occurs in the cell and acetate groups are removed (**Figure 5**)[16].

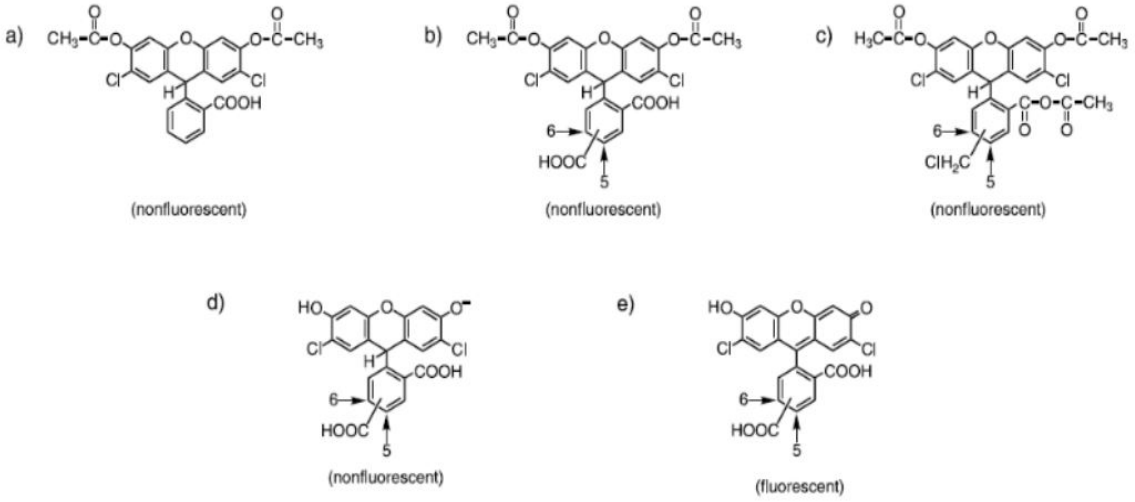


Figure 5. Structures of nonfluorescent **(a-d)** and acetylated forms of DCF **(e)**[16]

Oxidative stress in the mitochondria will be determined using the MitoTracker Red dye[17]. Mitochondria Peroxy Yellow 1 (MitoPY1) uses a triphenylphosphonium portion of the molecule to detect the mitochondria, and then undergoes a "boronate-based" (**Figure 6**) switch upon oxidation detected in the mitochondria[18].

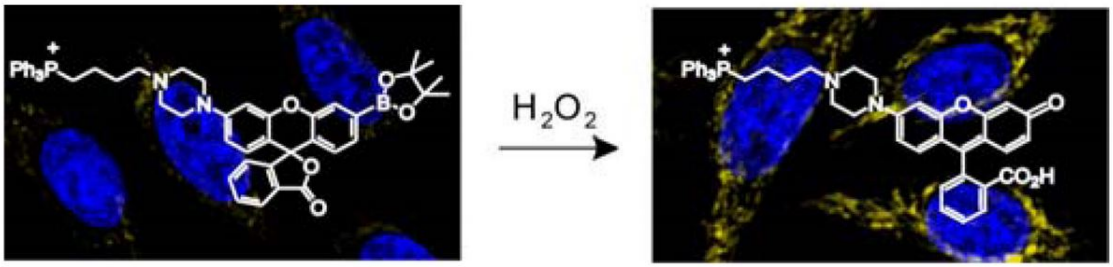


Figure 6. "Boronate-based" switch of MitoPY1 upon oxidation with hydrogen peroxide[18]

Oxidation of the probes will be measured using flow cytometry to quantify levels of ROS in the cytoplasm, as well as in the mitochondria. This will give more insight to ACM-induced oxidative changes in human B-leukemia cells.

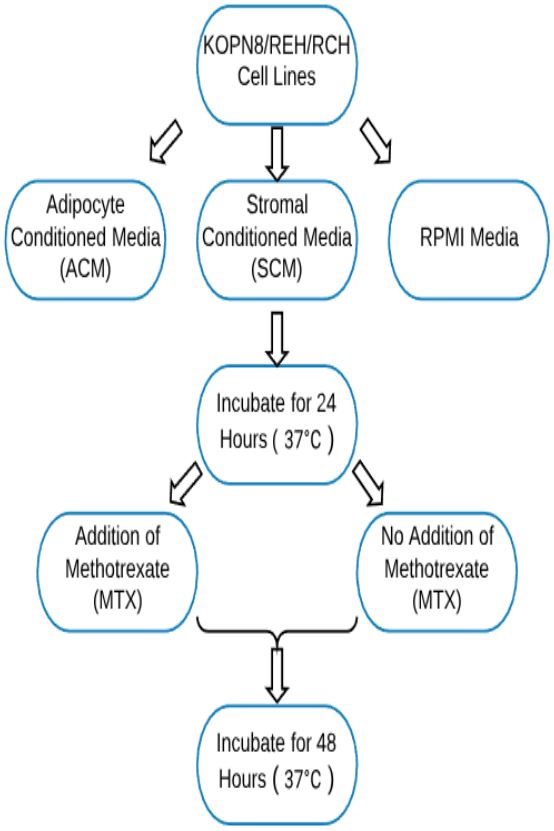
We will perform qPCR analysis to confirm that genes of interest were increased or decreased in B-leukemia cells exposed to ACM. After "hits" are confirmed, we will explore the impact of manipulating these genes on ACM-induced chemoresistance in human B-leukemia cells.

Although the adipocytes used in these studies were from mice, the next steps are to use human adipocytes to see if these findings are elevated, reduced, or remain constant. Studies have identified similar effects of mouse adipocytes on human and mouse leukemia cells but using human leukemia cells and human adipocytes is the next step in the solidification of these data[19]. We will also determine if obesity attenuates the efficacy of chemotherapies in murine models using flow cytometry, qPCR, and Western blot-based approaches.

5) Experimental

5.1) General Remarks

Prior to each experiment, KOPN8, REH, and RCH cell lines were each incubated with either ACM, SCM, or RPMI, as shown in **Scheme 2**. Experiments involving drug required an additional incubation step. These included experiments for cell cycle data, apoptosis, and RNA-sequencing.



Scheme 2. General experimental procedures for cells in ACM, SCM and RPMI both with and without MTX

5.2) Cell Cycle Data and EdU Analysis

EdU staining was done following the protocol from Invitrogen (Molecular Probes)[20]. Cells were labeled to obtain a final concentration of 10 μM EdU using a 10mM stock EdU. 5 μL of 200x were added into each well of a 12 well plate. 100 μL of Click-iT fixative were added to each well, then incubated for 15 minutes at room temperature. The plate was covered from the light to prevent denaturing. Cells were then washed with 1% BSA in PBS, centrifuged, with the supernatant removed. Cells were resuspended in 100 μL of 1X Click-iT saponin-based permeabilization and washed, then incubated for 15 more minutes. They were then washed again with 1% BSA in PBS. 50 μL of Click-iT EdU cocktail (43.8 μL 1X PBS, 1 μL $CuSO_4$, 0.25 μL fluorescent dye azide, and 5 μL of reaction buffer additive) was added to each, then incubated for 30 minutes at room temperature, again protected from light. Cells were washed again with the 1X Click-iT saponin-based permeabilization, washed, and had the supernatant removed. The appropriate DNS stains were added to each tube.

5.3) Apoptosis

Annexin V-FITC/PI was performed to determine levels of apoptosis in each cell line. Cells were washed with 1X PBS and resuspended in 200 μL of 1X binding buffer (made by adding 50 mL binding buffer to 150 mL distilled water). 5 μL of Annexin V-FITC was added to 195 μL of solution. The cells were incubated for 10 minutes at room temperature. Cells were washed in 200 μL of 1X binding buffer and resuspended in 190 μL 1X binding buffer. 10 μL of propidium iodide were added (stock 20 μg/mL). Methotrexate was added after 24 hours of incubation with the specific conditioned media. FACS analysis was done.

5.4) Changes in Gene Expression Profiles and RNA Sequencing

Cells were incubated in media (**Scheme 2**) for 48 hours total. After the initial 24-hour incubation, MTX was either added or not added, followed by an additional incubation period of 24 hours (rather than 48 hours as indicated in the diagram). This was to ensure that gene expression profiles were obtained for living cells prior to the induction of cell death.

Human B-leukemia cells (10^6) were treated with unconditioned media (DMEM), stromal cell-conditioned media (SCM), and adipocyte-conditioned media (ACM) in the presence and absence of MTX (50 mM) for 24 hours prior to harvesting cells for RNA isolation. RNA was isolated using the RNeasy® Mini Kit per protocol instructions (Qiagen; catalog no. 74104). Once isolated, samples were frozen and submitted for sequencing (in collaboration with Dr. Christopher Scharer; J.M. Boss Laboratory, Emory University). Samples were run on an iLLumina® NextSeq 500 instrument. From this experiment over 20 million unique reads were detected, 8 million reads were duplicates, and 2 million reads were unmapped. Overall, 13,198 genes were detected.

Mass spectrometry analysis of unconditioned media, stromal cell-conditioned media (SCM), and adipocyte-conditioned media (ACM) uses peptide fragments to reconstruct proteins based off of annotated sequence homology. This approach was used to identify components in the unconditioned media, stromal-cell conditioned media, and adipocyte-conditioned media. Our initial experiment was performed without trypsinization which allows for the detection of smaller proteins and peptides, and subsequent studies will be performed with trypsinization to identify larger proteins that are secreted by adipocytes. The top ten detected proteins in unconditioned media, SCM, and ACM are shown in **Figure 1**.

6) References

1. Jemal, A.; Siegel, R.; Xu, J.; Ward, E., Cancer statistics, 2010. *American Cancer Society* **2010**, *60,* 277-300. doi:10.1002/caac.20073.

2. Pui, C., Childhood leukemias. *The New England Journal of Medicine* **1995***, 332(24),* 1618-1630.

3. Behan, J. W.; Yun, J. P.; Proektor M. P.; Ehsanipour E. A.; Arutyunyan A.; Moses, A. S.; Avramis, V. I.; Louie, A. G.; Butturini, A.; Heisterkamp, N.; Mittelman, S. D., Adipocytes impair leukemia treatment in mice. *Cancer Research* **2009**, *69(19),* 7867-7874. doi: 10.1158/0008-5472.CAN-09-0800.

4. Orgel, E.; Tucci, J.; Alhushki, W.; Malvar, J.; Sposto, R.; Fu, C. H.; Freyer, D. R.; Abdel-Azim, H.; Mittelman S. D., Obesity is associated with residual leukemia following induction therapy for childhood B-precursor acute lymphoblastic leukemia. *Blood* **2014**, *124(26),* 3932-3938. doi:10.1182/blood-2014-08-595389.

5. Sheng, X.; Parmentier, J.; Tucci, J.; Pei, H.; Cortez-Toledo, O.; Dieli-Conwright, C. M.; Oberley, M. L.; Neely, M.; Orgel, E.; Louie, S.G.; Mittelman, S. D., Adipocytes sequester and metabolize the chemotherapeutic daunorubicin. *American Association for Cancer Research* **2017***,* 1-10. doi: 10.1158/1541-7786.MCR-17-0338.

6. Daldrup-Link, H. E.; Henning, T.; Link, T. M., MR imaging of therapy-induced changes of bone marrow. *European Radiology,* **2007**, *17,* 743-761. doi: 10.1007/s00330-006-0404-1.

7. Basen-Engquist, K.; Chang, M., Obesity and cancer risk: recent review and evidence. *Curr Oncol Rep,* **2011**, *13(1):* 71–76. doi:10.1007/s11912-010-0139-7.

8. Sheng, X.; Tucci, J.; Parmentier, J.; Ji, L.; Behan, J. W.; Heisterkamp, N.; Mittelman, S. D., Adipocytes cause leukemia cell resistance to daunorubicin via oxidative stress response. *Oncotarget* **2016**, *7(45),* 147-159.

9. Ehsanipour, E. A.; Sheng, X.; Behan, J. W.; Wang, X.; Butturini, A.; Avramis, V. I.; Mittelman, S. D., Adipocytes cause leukemia cell resistance to L-asparaginase via release of glutamine. *American Association for Cancer Research* **2013**, *73(10),* 2998-3006. doi: 10.1158/0008-5472.CAN-12-4402.

10. Aslanian, A. M.; Kilberg, M. S, Multiple adaptive mechanisms affect asparagine synthetase substrate availability in asparaginase-resistant MOLT-4 human leukaemia cells. *Biochemical Society* **2001**, *358,* 59-67.

11. Hino, M., Nakao, T., Yamane, T., Ohta, K., Takubo, T., & Tatsumi, N. (2000). Leptin receptor and leukemia. *Leukemia & Lymphoma* **2007**, *36:5-6*, 457-461.

12. Chen, J., Multiple signal pathways in obesity-associated cancer. *International Association for the Study of Obesity* **2011***, 12,* 1063-1070.

13. Konopleva, M.; Mikhail, A.; Estrov, Z.; Zhao, S.; Harris, D.; Sanchez-Williams, G.; Kornblau, S. M.; Dong, J.; Kliche, K. O.; Jiang, S.; Snodgrass, H. R.; Estey, E. H.; Andreeff, M, Expression and function of leptin receptor isoforms in myeloid leukemia and myelodysplastic syndromes: proliferative and anti-apoptotic activities. *Blood* **1999***, 93(5),* 1668-1676.

14. Yun, J. P.; Behan, J.W.; Heisterkamp, N.; Butturini, A.; Klemm, L.; Ji, L.; Groffen, J.; Müschen, M.; Mittelman, S.D., Diet-induced obesity accelerates acute lymphoblastic leukemia progression in two murine models. *Cancer Prev Res,* **2010**, *3(10)*, 1259-1264.

15. ThermoFisher Scientific; CellROX® oxidative stress reagents*,* **2012,** Pub. No. MP10422.

16. ThermoFisher Scientific; Reactive oxygen species (ROS) detection reagents*,* **2006**, Pub. No. MAN0002456.

17. ThermoFisher Scientific; MitoTracker mitochondria-selective probes*,* **2008**, Pub. No. MP 07510.

18. Dickinson, B. C.; Chang, C. J., A targetable fluorescent probe for imaging hydrogen peroxide in the mitochondria of living cells. *J Am Chem Soc*, **2008**, *130(30),* 9638–9639. doi:10.1021/ja802355u.

19. Hagberg, C. E.; Li, Q.; Kutschke, M.; Bhowmick, D.; Kiss, E.; Shabalina, I. G.; Harms, M. J.; Shilkova, O.; Kozina, V.; Nedergaard, J.; Boucher, J.; Thorell, A.; Spalding, K. L., Flow cytometry of mouse and human adipocytes for the analysis of browning and cellular heterogeneity. *Cell Reports,* **2018,** 24(10): 2746–2756. doi: 10.1016/j.celrep.2018.08.006.

20. ThermoFisher Scientific; EdU–A superior BrdU alternative for cell proliferation, MAN0009883.