Anti-apoptotic *BCL2L2* increases megakaryocyte proplatelet formation in cultures of human cord blood

Seema Bhatlekar,[1] Indranil Basak,[1] Leonard C. Edelstein,[2] Robert A. Campbell,[1] Cory R. Lindsey,[2] Joseph E. Italiano Jr.,[3] Andrew S. Weyrich,[1] Jesse W. Rowley,[1] Matthew T. Rondina,[1,4] Martha Sola-Visner[5] and Paul F. Bray[1,6]

[1]Program in Molecular Medicine and Department of Internal Medicine, University of Utah, Salt Lake City, UT; [2]Cardeza Foundation for Hematologic Research, Thomas Jefferson University, Philadelphia, PA; [3]Brigham and Women's Hospital, Harvard University, Boston, MA; [4]George E. Wahlen VAMC GRECC, Salt Lake City, UT; [5]Boston Children's Hospital, Harvard University, Boston, MA and [6]Division of Hematology and Hematologic Malignancies, Department of Internal Medicine, University of Utah, Salt Lake City, UT, USA

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Correspondence: *PAUL F. BRAY* - paul.bray@hsc.utah.edu

Supplemental Data

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Supplemental methods

Primary cell culture. Human umbilical cord blood obtained from the New York Blood Center (New York, NY) under institutional review boards (IRB) approval (00108527), diluted 1:2 with PBS and layered it over Ficol/Histopaque (Sigma, St. Louis, MO). After 30 minutes of centrifugation at 400x *g*, buffy coat was collected. CD34-positive cells were isolated using magnetic immunoselection following the manufacture's protocol (MiltenyiBiotec, Auburn, CA). Freshly isolated CD34-positive cells were cultured at $1x10^6$ cells/mL (termed "day 0" cells) in StemSpan SFEM II medium (Stemcell Technologies, Vancouver, BC) supplemented with 25 ng/mL SCF and 20 ng/mL TPO (Peprotech, Rocky Hill, NJ). Every 3 days, cells were harvested, counted and re-plated at a density of $1x10^6$ cells/mL with fresh medium until day 6. At day 6, culture was supplemented with only 50 ng/mL TPO in fresh StemSpan SFEM II medium at a density of $5x10^5$ cells/mL. Every 3 days, cells were harvested, counted and re-plated at a density of $5x10^5$ cells/mL with fresh medium and cell were cultured for 13 days.

For experiments using adult CD34+ cells, GM-CSF mobilized CD34+ cells were purchased from the Utah Cell Therapy and Regenerative Medicine Center (Salt Lake City, UT) under the University of Utah IRB approval (00108527). All donors were

healthy, provided informed consent and authorized the utilization of their cells for research in the event that they no longer needed them. Adult CD34+ cells were cultured similar to umbilical cord blood to generate megakaryocytes in cultures for 13 days.

Cells were harvested at day 13 and MK differentiation was assessed by ploidy [1] and flow cytometry by monitoring expression of CD61, CD41a, CD42a and CD42b (BD Pharmingen, San Diego, CA).[2] Unless otherwise stated, all assays were performed using day 13 cultures MKs.

Annexin V staining. Annexin V was used to identify the phosphatidylserine (PS) expressing cells as a marker of apoptosis.[3] Day 13 cultured cells were washed with cold PBS (2.7 mM KCl, 1.47 mM KH_2PO_4, 138 mM NaCl, 8 mM Na_2HPO_4-$7H_2O$) and resuspended in 100 µL 1x annexin V binding buffer (BD Biosciences, Franklin Lakes, NJ), stained with 5 µL PE-labeled annexin V (BD Biosciences) and APC-labeled anti-CD41a (1:50) or APC-conjugated IgG for 15 minutes at room temperature in the dark. Unstained cells were used as control for gating on a Cytoflex flow cytometer (Beckman Coulter, Inc., Brea, CA).

Annexin V Microbead Purification. Cells were resuspended in 1x annexin V binding buffer. Annexin V microbeads were added as per manufacturer's instructions (MiltenyiBiotec, Auburn, CA), incubated for 15 minutes at 4°C, and washed and resuspended in 1x annexin V binding buffer. PS^{Low} LLG and PS^{High} SHG cells were separated using an LS column (MiltenyiBiotec).

Transmission Electron Microscopy. The procedure was performed at the Thomas Jefferson University, Electron Microscopy Core Facility, as previously described.[4] Briefly, purified PS^{Low} LLG and PS^{High} SHG cells were fixed in 2% buffered glutaraldehyde and post-fixed in 1% buffered osmium tetroxide. Following embedding and preparing of thick sections, 80 nM sections were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and imaged in a JEM-100CX II transmission electron microscope (JEOL Ltd., Tokyo).[4]

Phalloidin staining. PS^{Low} and PS^{High} cells were plated on fibrinogen coated 8 well chamber slide in fresh SFEM media supplemented with 50 ng/ml TPO and incubated overnight at 37˚C. Media was removed and cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes followed by PBS washing. Blocking was performed for 30 minutes at 37˚C (1% BSA + 10 % normal goat serum + 0.5 %Tween 20 in PBS). Alexa flour 488 Phalloidin and Alexa fluor 594 Wheat Germ Agglutinin (WGA) (1:200, Life technologies) was added on to the cells and incubated for 1 hour at room temperature in dark. Cells were washed with PBS and stained with TOPRO dye (1:1000, Thermo Fischer Scientific, Waltham, MA) for 5 minutes. Cells were washed with PBS and imaged under EVOS cell imaging systems (Thermo Fisher Scientific).

Tetramethylrhodamine, methyl ester (TMRM) staining. Cells were stained with APC-labeled anti-CD41a (1:50) by incubating at 37°C for 15 minutes, followed by

incubation with TMRM dye (50nM) (Thermo Fisher Scientific) at 37°C, 5% CO_2 for 45 minutes. Cells were analyzed using a Cytoflex flow cytometer.

Measurement of Ca^{2+} mobilization. Cells were incubated with the cell-permeable Ca^2+ sensitive dye Fluo-4 AM (0.5 μg/μL) (Invitrogen, Carlsbad, CA) for 10 minutes at 37°C, recalcified to a final concentration of 1 mM $CaCl_2$, and stimulated with 250 nM thrombin or no agonist (resting) as control. Changes in intracellular Ca^{2+} flux were monitored over 6 minutes using BD Accuri C6 flow cytometer as we have previously described.[2]

DiO labeling. PS^{Low} LLG cells were separated from PS^{High} SHG cells by annexin V microbeads, labeled with Vybrant DiO cell tracking dye (Life Technologies), washed twice with Tyrode's buffer and re-cultured for 2 days in StemSpan II SFEM medium supplemented with 50 ng/mL TPO. At day 15, cells were analyzed using BD Accuri C6 flow cytometer. Day 13 cells without DiO labeling were used as control for gating.

RNA sequencing. CD61+ MKs were isolated on day 6 and day 13 cultures using anti-CD61 immunomagnetic beads as per manufacturer's instructions (Milltenyi Biotec). RNA was extracted using the Total RNA Purification kit (Norgen Bioteck, Inc. Ontario). cDNA synthesis was performed using the Ovation® RNA-Seq System V2 (NuGEN Technologies, Inc. San Carlos, CA). The amplified cDNA was used for library preparation using the Ovation Ultralow System V2 (NuGEN Technologies). Paired end RNA sequencing and analysis was performed at the Children's Hospital of Philadelphia,

Center for Applied Genomics.[5] Alignments were generated using the ultrafast universal RNA-Seq aligner (STAR). Differential expression tests were performed using the cuffdiff package in cufflink 2.2.1 and the GTF file from GENCODE version 19, using only non-ribosomal RNA alignment reads.

Real time PCR quantification of *BCL2L2*. For validation of RNA-seq candidates, CD61+ MKs were isolated using CD61 microbeads (MiltenyiBiotec) on day 6 and 13 in cultures. For assessing *BCL2L2* changes in culture, LLG and SHG cells were sorted based on the forward and side scatter characteristics on a BD FACSAria cell sorter (Flow Cytometry Core, Sidney Kimmel Cancer Center at Thomas Jefferson University, Philadelphia, PA) on day 6, 9 and 13 cultures. RNA was isolated using Trizol (Life Technologies) and cDNA was prepared as per manufacture's protocol (Invitrogen). SYBR green qPCR assay (Thermo Fisher Scientific) was performed using primers: BCL2L2 F 5'-GTGCTGTGTCTTAAGAGCTGCCAT-3'; BCL2L2 R 5'-GACATAA CCCTTCTGCCTCAGCTT-3'; BAX F 5'- GCTCTGAGCAGATCATGAAGAC-3' BAX R 5'-GCTTCTTGGTGGACGCAT-3'; BAK1 F 5'- GTTTTCCGCAGCTACGTTTTT-3'; BAK1 R 5'- GCAGAGGTAAGGTGACCATCTC-3'; BCL2L1 F 5'-GAGCTGGTGGTTGACTTTCTC-3'; BCL2L1 R 5'- TCCATCTCCGATTCAGTCCCT-3'; 18srRNA F 5'-CGGCTACCACATCCAAGG-3'; 18srRNA R 5' GCTGCTGGCACCAGACTT-3; GAPDH F 5'- ACCGTCAAGGCTGAGAAC-3'; GAPDH R 5'-TTCTCCATGGTGGTGAAGAC-3'.

Lentiviral constructs, isolation and cell transduction. We modified our prior approach to transduce cultured MKs[1] by using more effective lentiviral vectors. Green fluorescent protein (GFP) was cloned into gene expression vector pCDH driven by MSCV promoter (pCDH-MSCV-GFP; System Biosciences, Palo Alto, CA). The human *BCL2L2* coding sequence was cloned into pCDH-MSCV-GFP lentiviral vector (System Biosciences) at NheI and NotI sites by PCR-based strategy using platelet cDNA as template. Primers used were: *BCL2L2*-EcoRI-F 5'- GGAATTCATGGCGACCC CAGCCTCGGC -3', *BCL2L2*-NotI-R 5'-ATAAGAATGCGGCCGCTCACTTGC TAGCAAAAAAGGCCCC-3'. All constructs were validated by DNA sequencing. The pCDH-MSCV-*BCL2L2* was co-transfected with the packaging vector pCMVR8.74 and the envelope vector pMD2.G (System Biosciences) into the 293Ta cell line (GeneCopoeia, Rockville, MD) using Lipofectamine 2000 (Invitrogen), and lentiviral particles were collected for 3 days. Lentiviral titer and precipitation was determined as done previously.[6] *BCL2L2* lentiviral particles were transduced (MOI of 5) in cultures at day 5. The lentiviral vector encoding only GFP (empty vector control) was used as a negative control. The transduced cells were cultured in SFEM medium supplemented with cytokines and cultured as described.[2] Day 13 fluorescence data was acquired using a BD Accuri C6 or Cytoflex flow cytometer.

Western blotting. Cells transduced with *BCL2L2* and control lentiviral vectors were lysed with 1x cell lysis buffer (Cell Signaling Technology, Danvers, Massachusetts) and analyzed by western immunoblotting.[2] Total cell lysate (30 µg) was separated by SDS polyacrylamide gel electrophoresis, electrotransferred onto PVDF membranes, blocked

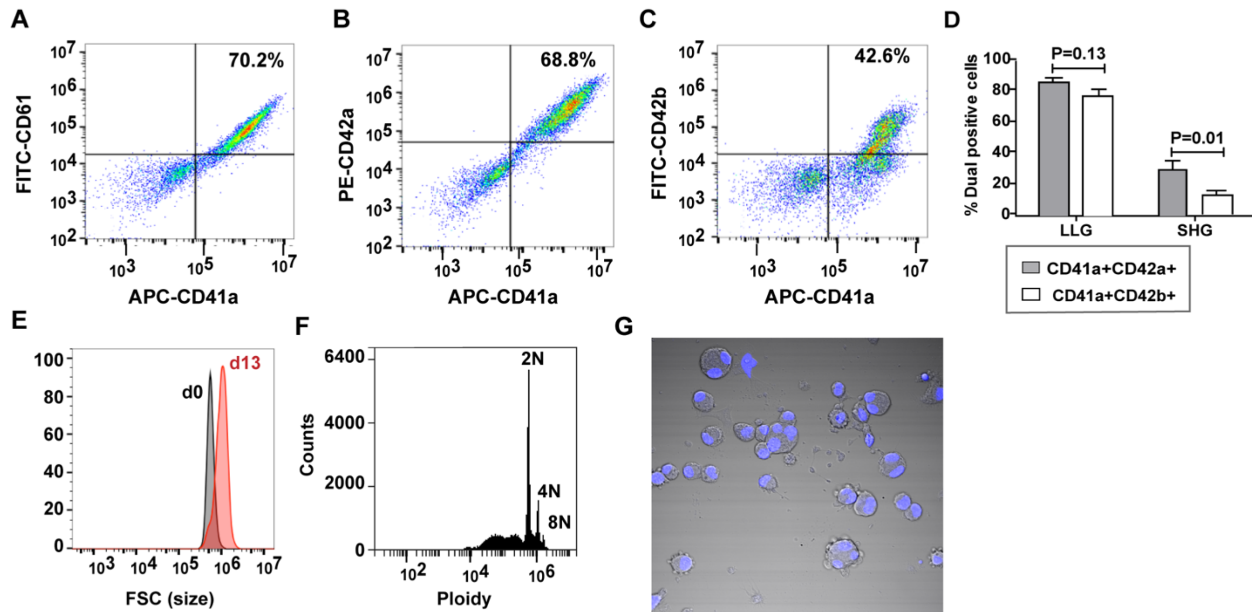
in TBS, 0.1% Tween 20 and 5% dry milk and immunoblotted with anti-BCL2L2 (1:100) (Abcam, Cambridge). Anti-GAPDH Ab (1:2000, Santa Cruz Biotechnology, Dallas, TX) was used as a control for protein loading and densitometric quantification. Samples with both RNA and protein were used for analysis.

Tubulin staining. Transduced cells were placed at 1×10^4 cells/ml on immobilized human fibrinogen (100 µg/ml; Calbiochem-Novabiochem Corporation, San Diego, CA) in 8-well chamber slide using a fresh medium supplemented with 50 ng/mL TPO. Tubulin staining was performed as described.[2] Human anti-α-tubulin (1:100) (Cell Signaling Technology) was used as a primary antibody and Alexa Fluor 568 (1: 5,000) (Thermo Fisher Scientific) was used as a secondary antibody. Nuclei were stained with DAPI (1:5000) (Molecular Probes, Eugene, OR). Images were taken at room temperature under 40x objective using FV1000 confocal laser scanning microscope. Images were further analyzed using ImageJ (National Institute of Health, Bethesda, Maryland). Background light was adjusted differently for *BCL2L2* and Ctrl image to account for background light gradient.

Cultured platelet-like particle isolation, staining and agonist activation. Cultured platelet-like particles (PLPs) were isolated by density centrifugation at day 13 as described.[7] PLPs were stained with APC-labeled anti-CD41a (1:50) for 15 minutes at 37°C. For PAC1 and anti-P-Selectin binding, PLPs were stained with PE-labeled anti-CD42a (1:100) and APC-labeled PAC1 (1:100) (BioLegend, San Diego, CA) or with APC-labeled anti-CD41a (1:50) and PE-labeled anti-P-Selectin (1:100) respectively in

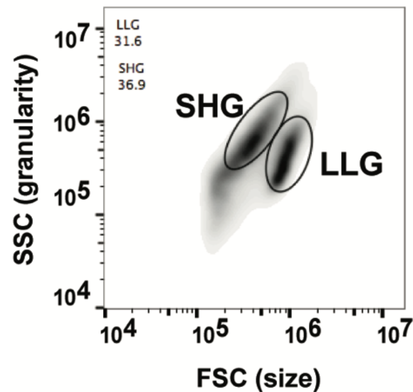
response to stimulation with no agonist (resting) or 250 nM thrombin for 20 minutes at 37°C followed by 4% paraformaldehyde fixation. IgG and IgM isotype controls were used to assess non-specific background. Thrombin activated cells were analyzed for PAC1 and anti-P-selectin antibody binding in CD42a+ and CD41a+ cells, respectively. Fluorescence data was acquired on a Cytoflex flow cytometer. Data is presented after subtraction of background fluorescence obtained from unstimulated (resting) cells.

Supplemental figures

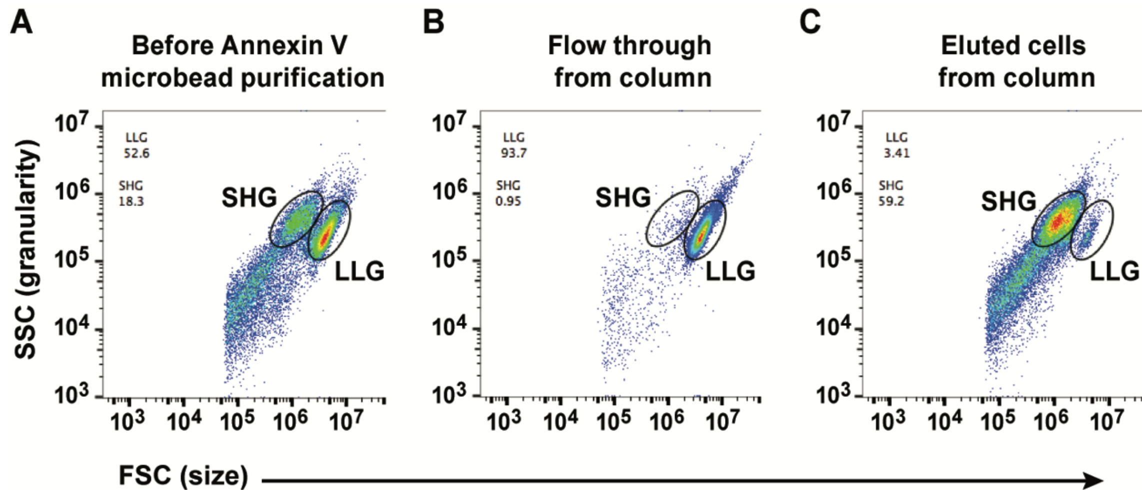


Supplemental Figure 1. Human umbilical cord blood CD34+ derived megakaryocyte cultures. Representative flow cytometric analysis for megakaryocyte marker expression (**A-C**). Dual staining of cultured day 13 cells with **(A)** FITC-labeled anti-CD61 and APC-labeled anti-CD41a or **(B)** PE-labeled anti-CD42a and APC-labeled anti-CD41a or **(C)** FITC-labeled anti-CD42b and APC-labeled anti-CD41a. **(D)** Quantification of mature MKs (defined by CD41a+CD42a+ and CD41a+CD42b+) in LLGs and SHGs for day 13 cultured cells (n=10). Shedding of CD42b (GPIbα) was significantly higher than CD42a in apoptotic SHGs. **(E)** Representative histogram of cell size based on forward scatter (FSC) for day 13 cultured cells (red) compared to day 0 (d0) CD34+ HSCs (grey). **(F)** Representative ploidy analysis for day 13 cultured cells. Note: We did not gate out the sub-2N cells. **(G)** Polyploid MKs are readily identified by

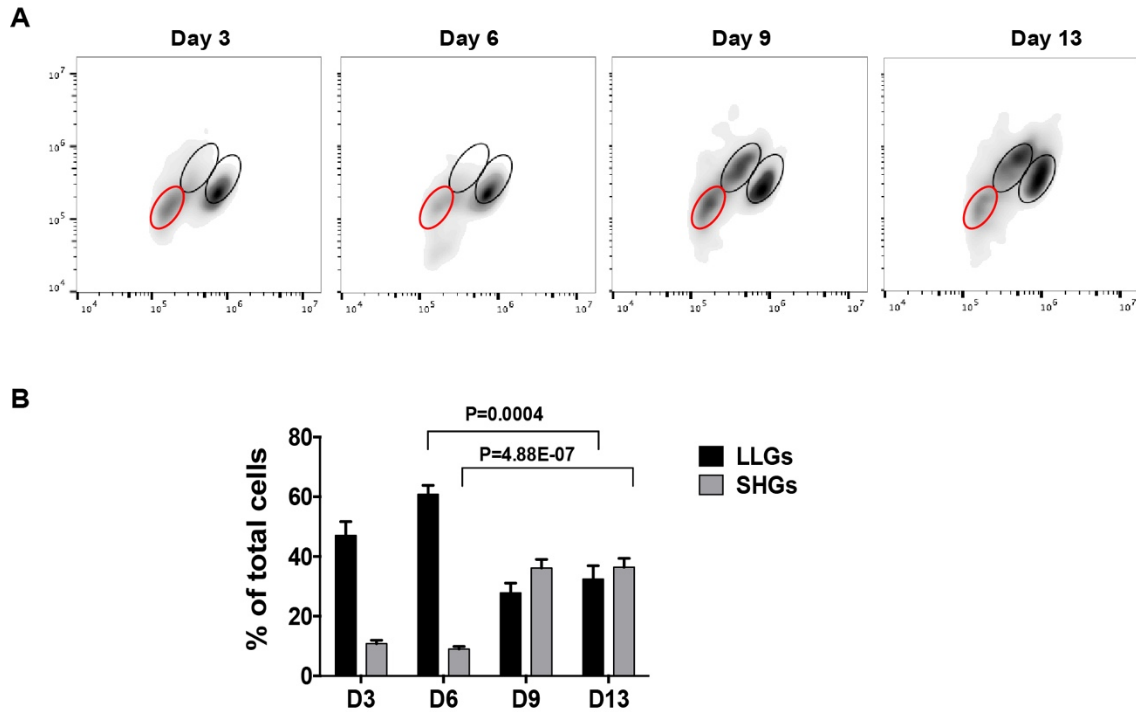
DAPI stain. Blue color indicates nuclear stain, DAPI with brightfield background. Image was taken under 40x objective using FV1000 confocal laser scanning microscope (Olympus, Center Valley, PA).



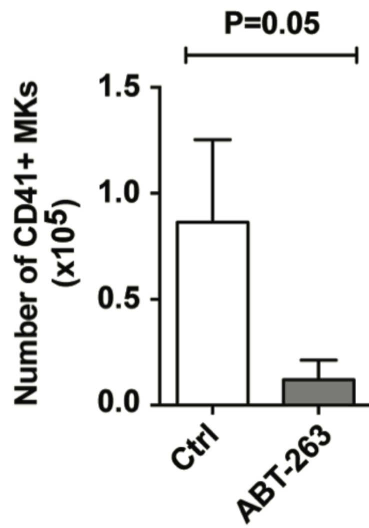
Supplemental Figure 2. Adult CD34+ derived megakaryocytes analyzed by flow cytometric forward scatter (FSC) and side scatter (SSC). CD34+ HSCs isolated from adult peripheral blood were cultured to generate megakaryocytes using 25 ng/mL stem cell factor (SCF) and 20 ng/mL thrombopoietin (TPO) until day 6. Fifty ng/mL TPO was used from day 6 to 13. Media was replenished with cytokines every 3 days. On day 13, cells were analyzed for forward scatter (FSC) and side scatter (SSC) using a Cytoflex flow cytometer. Similar to cord blood-derived MKs, LLG and SHG MKs were observed.



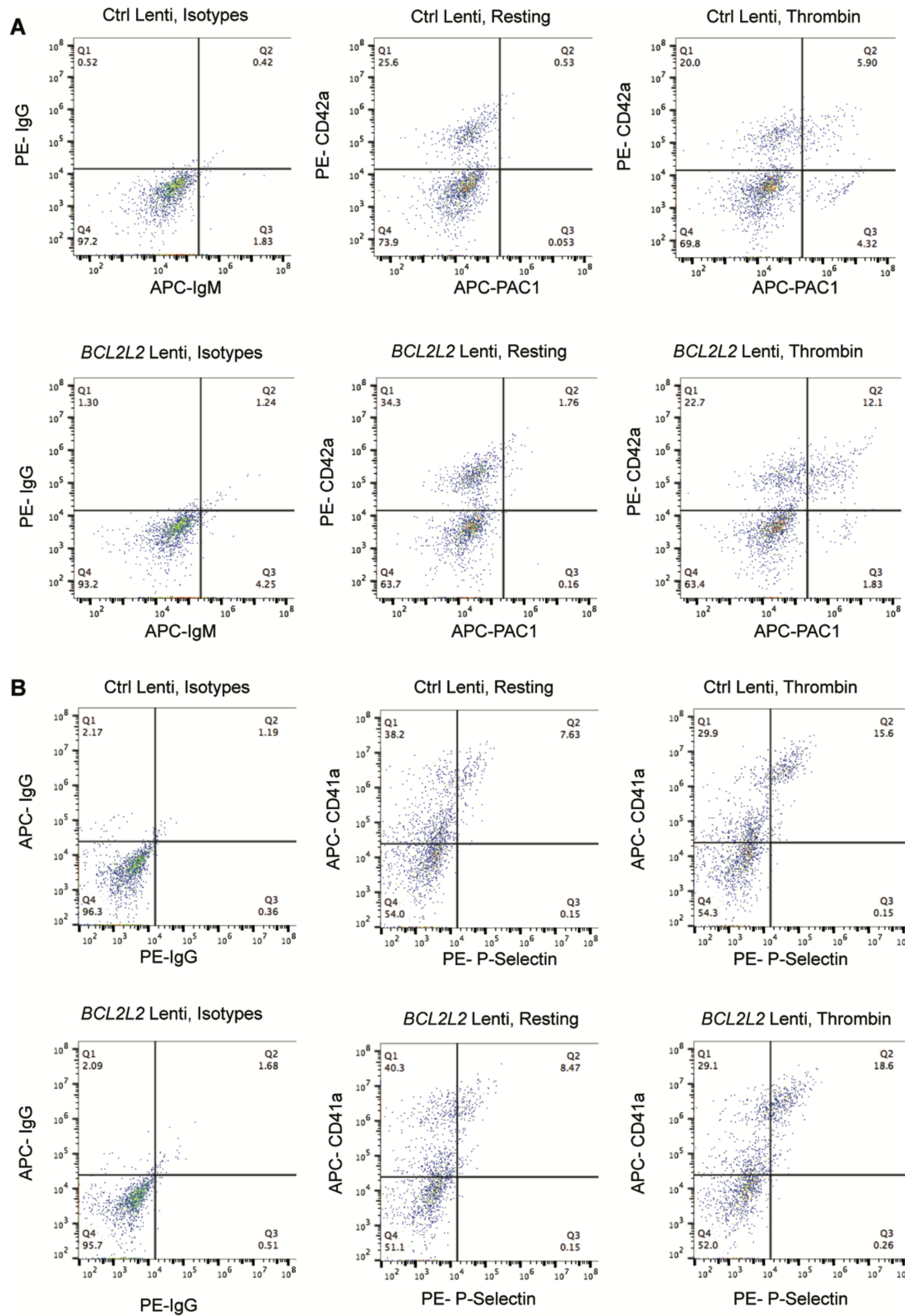
Supplemental Figure 3. **Purity of LLG and SHG populations after isolation based on phosphatidylserine expression.** Day 13 cultured cells were labeled with annexin V microbeads and PS^{Low} LLG megakaryocytes were separated from PS^{High} SHGs. Representative flow cytometry data showing LLG and SHG megakaryocytes at day 13 **(A)** before annexin V microbead purification, **(B)** the flow-through from column that did not bind annexin V isolating PS^{Low} LLG megakaryocytes, and **(C)** the eluted cells from column bound to annexin V that was predominantly PS^{High} SHG megakaryocytes.



Supplemental Figure 4. Characterization of appearance of LLGs and SHGs in cultures over time. **(A)** Representative image showing appearance of LLGs and SHGs over time in culture. The black ovals indicate the SHG and LLG population. The red oval indicates the contaminating platelets (day 3) and platelet-like particles (days 6-13). **(B)** Quantification of LLGs and SHGs relative to total cells in cultures at day 3, 6, 9 and 13. LLGs decreased significantly from day 6 to day 13 while SHGs significantly increased from day 6 to day 13 in cultures (n=12).

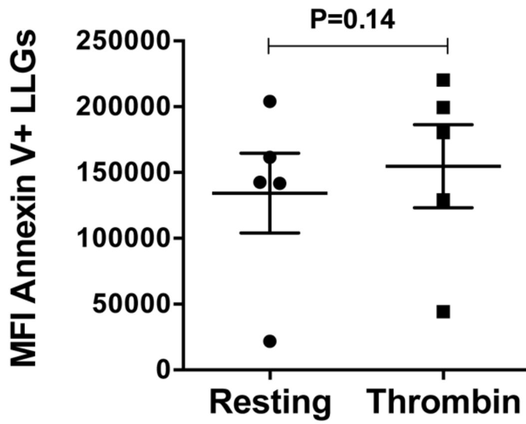


Supplemental Figure 5. Effects of ABT-263 treatment on cultured megakaryocytes. Bcl-2 family member inhibitor, ABT-263 (Navitoclax; 100 nM) (Selleckchem, Houston, Texas) was added to day 5 cultures and replenished with 100 nM ABT-263 every three days. Day 13 cells were stained with APC-labeled anti-CD41a or APC-conjugated IgG, and the number of CD41a+ megakaryocytes was analyzed using a Cytoflex flow cytometer (n=6). Untreated cells cultured similar to ABT-263 treated cells were used as a negative control (Ctrl).

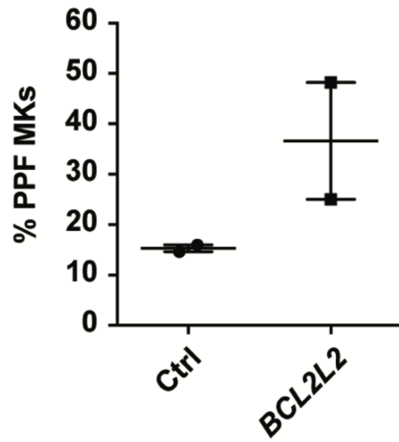


Supplemental Figure 6. Flow cytometric analysis of platelet like particles on thrombin stimulation. Day 13 cultured platelet like particles were isolated after megakaryocyte transduction with empty lentiviral vector (Ctrl Lenti) or lentiviral vector

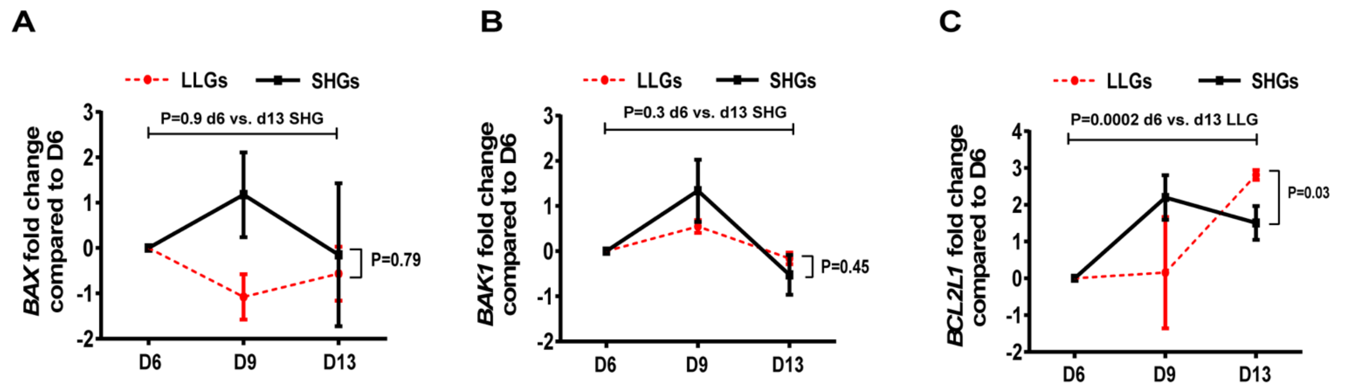
containing *BCL2L2 (BCL2L2* Lenti) and assessed for thrombin-stimulated PAC1 **(A)** or anti-P-selectin antibody **(B)** binding. **(A)** Representative flow cytometric analysis for platelet like particles isolated for Ctrl lenti and *BCL2L2* lenti, stained with APC labeled PAC1 and PE labeled anti-CD42a at resting (no agonist) or thrombin activated. APC labeled IgM and PE labeled IgG were used as isotype control for gating. **(B)** Representative flow cytometric analysis for platelet like particles isolated for Ctrl lenti and *BCL2L2* lenti, stained with PE labeled anti-P-Selectin and APC labeled anti-CD41a at resting (no agonist) or thrombin activated. PE labeled IgG and APC labeled IgG were used as isotype control for gating.



Supplemental Figure 7. Annexin V binding of thrombin stimulated LLGs. Day 13 cultured megakaryocytes were stained with PE labeled annexin V and FITC labeled PAC1, stimulated with 250 nM thrombin or at resting (no agonist) and analyzed by flow cytometer for annexin V+ LLGs. Mean fluorescence intensity (MFI) for annexin V+ LLGs were plotted for resting and thrombin stimulation (n=5).



Supplemental Figure 8. **Quantification of proplatelet forming megakaryocytes (PPF) in adult CD34 derived megakaryocytes on BCL2L2 overexpression.** CD34+ cells isolated from adult peripheral blood were transduced with empty lentiviral vector (Ctrl) or lentiviral vector containing *BCL2L2* (MOI=5) on day 5 and cultured for 13 days to generate megakaryocytes. Day 13 cells were quantified for proplatelet forming (PPF) MKs amongst total cells *blinded* as to treatment group (n=2).



Supplemental Figure 9. Expression of apoptosis genes in LLGs and SHGs. RNA was extracted from LLG and SHG MKs purified by flow cytometric cell sorting at day 6, day 9 and day 13. Substantial amounts of RNA were obtained from LLG cells; SHG cells yielded considerably less RNA, but still sufficient amounts to perform key qPCRs. Expression of (A) *BAX*, (B) *BAK1* and (C) *BCL2L1* was quantified by qPCR for both LLGs and SHGs. n=4 different cord blood MK cultures for day 6 and day 13 for all panels; n=6 for day 9 for panel A and n=4 for panel B and C.

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