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Regulation of Mcl-1 Expression in Context to Bone Marrow Stromal Microenvironment in Chronic Lymphocytic Leukemia[1]

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Abstract

A growing body of evidence suggests that the resistance of CLL cells to apoptosis is partly mediated through the interactions between leukemia cells and adjacent stromal cells residing in the lymphatic tissue or bone marrow microenvironment. Mcl-1, an anti-apoptotic protein that is associated with failure to treatment is up-regulated in CLL lymphocytes after interaction with microenvironment. However, the regulation of its expression in context to microenvironment is unclear. We evaluated and compared changes in Mcl-1 in CLL B-cells in suspension culture and when co-cultured on stromal cells. The blockade of apoptosis in co-cultured CLL cells is associated with diminution in caspase-3 and PARP cleavage and is not dependent on cytogenetic profile or prognostic factors of the disease. Stroma-derived resistance to apoptosis is associated with a cascade of transcriptional events such as increase in levels of total RNA Pol II and its phosphorylation at Ser2 and Ser5, increase in the rate of global RNA synthesis, and amplification of Mcl-1 transcript levels. The latter is associated with increase in Mcl-1 protein level without an impact on the levels of Bcl-2 and Bcl-xL. Post-translational modifications of protein kinases show increased phosphorylation of Akt at Ser473, Erk at Thr202/Tyr204 and Gsk-3β at Ser9 and augmentation of total Mcl-1 accumulation along with phosphorylation at Ser159/Thr163 sites. Collectively, stroma-induced apoptosis resistance is mediated through signaling proteins that regulate transcriptional and translational expression and post-translational modification of Mcl-1 in CLL cells in context to bone marrow stromal microenvironment.

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the gradual accumulation of mature, non-proliferative B-cells. High levels of anti-apoptotic protein Mcl-1 correlates with in vitro and in vivo chemo-responses and poor clinical outcome [1]. Elevated Mcl-1 levels prolonged the survival of CLL cells exposed to a variety of apoptosis-inducing stimuli; down-regulation of Mcl-1 using antisense oligonucleotides resulted in a significant cell death. Other approaches to alter Mcl-1 expression or its anti-apoptotic function using small molecule BH3 mimetics [2], peptidomimetics [3] or cyclin dependent kinases [4] predominantly sensitized CLL cells to programmed cell death. Together, these observations underscore Mcl-1 as an important prognostic factor in B-CLL pathogenesis.

A growing body of evidence suggests resistance of CLL cells to apoptosis is partly mediated by the interactions between leukemia cells and adjacent stromal cells residing in the lymphatic tissue or bone marrow microenvironment. The target surface-receptors on B-cell such as BCR (B-cell receptor) or CXCR4 are persistently activated by their respective ligands (anti-IgM or CXCL12) expressed on the stromal cells and or nurse like cells keeping the leukemia cells and stromal cells in homeostasis [5,6]. This signaling event leads to the activation of BCR

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signaling pathway, in which a number of downstream kinases are activated that are essential for survival, homing and retention of CLL cells [7]. In vitro studies using representative bone marrow stromal cells demonstrated that CLL cells co-cultured on stromal cells induced robust increase in anti-apoptotic protein Mcl-1, but not Bcl-2 or Bcl-xL and associated with CLL B-cell survival [8–10]. Other studies associated with co-culturing of CLL primary cells with representative lymph node microenvironment (with CD154-transduced system) demonstrated increase in Bcl-xL and Bcl2-A1, which are other anti-apoptotic members of Bcl-2 family proteins [11–13]. Activation of BCR pathway in a ligand-dependent manner significantly increased the levels of Mcl-1 in association with phosphorylation of Akt and Erk kinases [7]. Sustained activation of Akt, increased the level of pro-survival proteins Mcl-1, Bcl-xL and XIAP and enhanced the leukemia cell survival; however only down-regulation of Mcl-1, but not Bcl-xL or XIAP by siRNA treatment induced apoptosis of CLL cells demonstrating the direct association between Akt and Mcl-1 [6]. Spleen tyrosine kinase (SYK) in a ligand-independent BCR signaling, demonstrated its regulatory mechanism solely on Mcl-1 accumulation but not on XIAP [14]. Close association between the ability of anti-IgM to induce BIM phosphorylation and subsequent Mcl-1 release has been linked to progressive disease [15]. Collectively, these studies reveal that the proto-oncogenic role of Mcl-1 is not limited to sustained survival of circulating CLL lymphocytes, but extended to BCR-mediated apoptotic resistance in CLL. However, the mechanism or the molecular drive that induces the Mcl-1 in the microenvironment niches is unclear.

The regulation of Mcl-1 is a complex phenomenon as it is known to be regulated at different levels of transcriptional, translational and post-translational modifications. Expression of Mcl-1 is regulated by microRNAs miR15 and 16a, which are the negative regulators of Mcl-1. Activated *STAT3* is shown to bind in SIE related element in the murine Mcl-1 promoter and increase Mcl-1 protein levels [16]. Mcl-1 is also a direct target of many transcription factors such as *NF-κB* [17], *cMYC* [18], *CREB* [19], *STAT-5* [20]. Studies have previously revealed an increase in *NF-κB* [21] *and MYC* [22] when malignant cells were co-cultured with BMSC. The association between BCR protein kinases and Mcl-1 suggests that it is regulated by Akt and Erk kinases. Using NK-Tert bone-marrow stromal cell line we explore changes in key proteins in CLL lymphocytes. Our data show that the induction of Mcl-1 in bone marrow stromal microenvironment is associated with sequential events that include activation of global transcription, translation and post-translational (protein) modifications of signaling proteins.

Patients and Methods

The present study is carried out in leukemic lymphocytes isolated from peripheral blood samples obtained from patients with CLL as described previously [8]. All patients signed written informed consent forms in accordance with the Declaration of Helsinki and the laboratory protocol approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center.

Isolation of Normal Peripheral Blood Mononuclear Cells (PBMC) and B-Cells (CD19 + ve Cells)

Whole blood is collected from healthy donors under an institutional review board–approved protocol in heparinized tubes, and lymphocytes are separated using ficoll gradient method [8]. The isolated PBMCs are either used directly or subjected to B-cell enrichment kit from STEMCELL Technologies (catalog #19054) to purify CD19 positive B-cells using negative selection. The purity of B-cells is measured on the flow cytometer using CD19-APC antibody (before and after isolation).

Preparation of CLL-MSC Co-Cultures

Whole blood from CLL patients is collected in heparinized tubes, and lymphocytes are separated and cocultured with confluent layers of human bone marrow stromal cells (NKtert; RIKEN Cell Bank, Tsukuba, Japan) at a ratio of 100 CLL cells to 1 MSC or cultured alone [8]. The cell lines are routinely tested for *Mycoplasma* infection and authenticated by short tandem repeat analysis at MD Anderson Cancer Center's Characterized Cell Line Core facility [8].

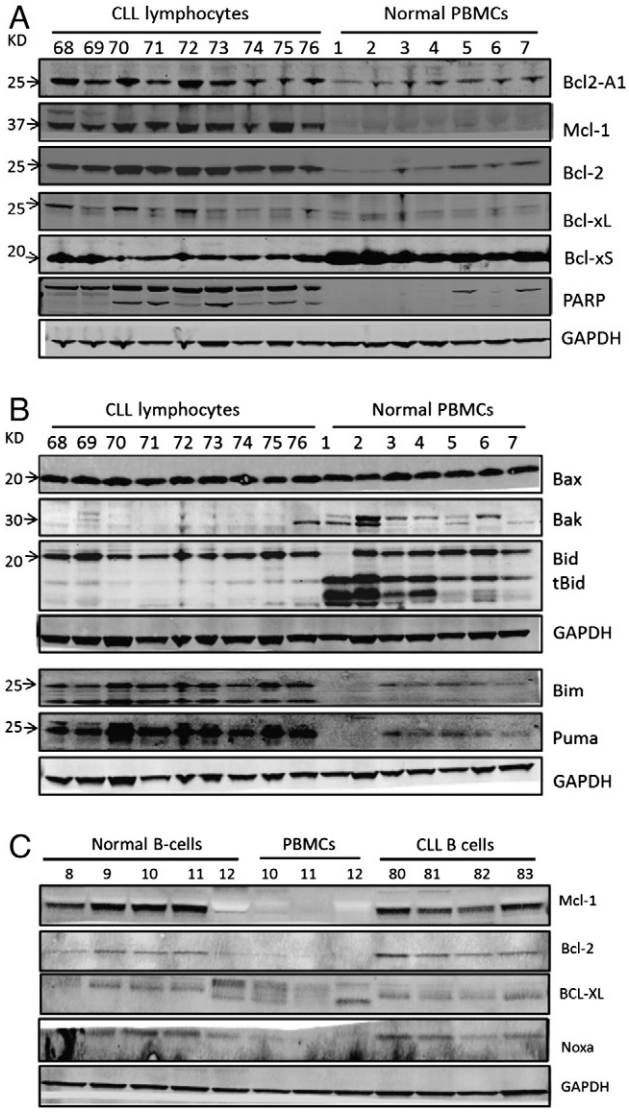


Figure 1. Comparison of expression of Bcl-2 family proteins in CLL lymphocytes, normal lymphocytes and normal B cells. Protein extracts obtained from CLL lymphocytes ($n = 9$) and normal lymphocytes ($n = 7$) are loaded in equal amounts side by side in the same gel and the levels of anti-apoptotic proteins (A) and pro-apoptotic proteins (B) are evaluated by immunoblotting analysis. GAPDH is used as a loading control. Normal B cells are isolated using B-cell enrichment kit by negative selection. Immunoblots for Mcl-1, Bcl-2, Bcl-xl and NOXA in isolated B cells ($n = 5$), normal PBMCSs ($n = 3$) and CLL lymphocytes ($n = 4$) are provided (C).

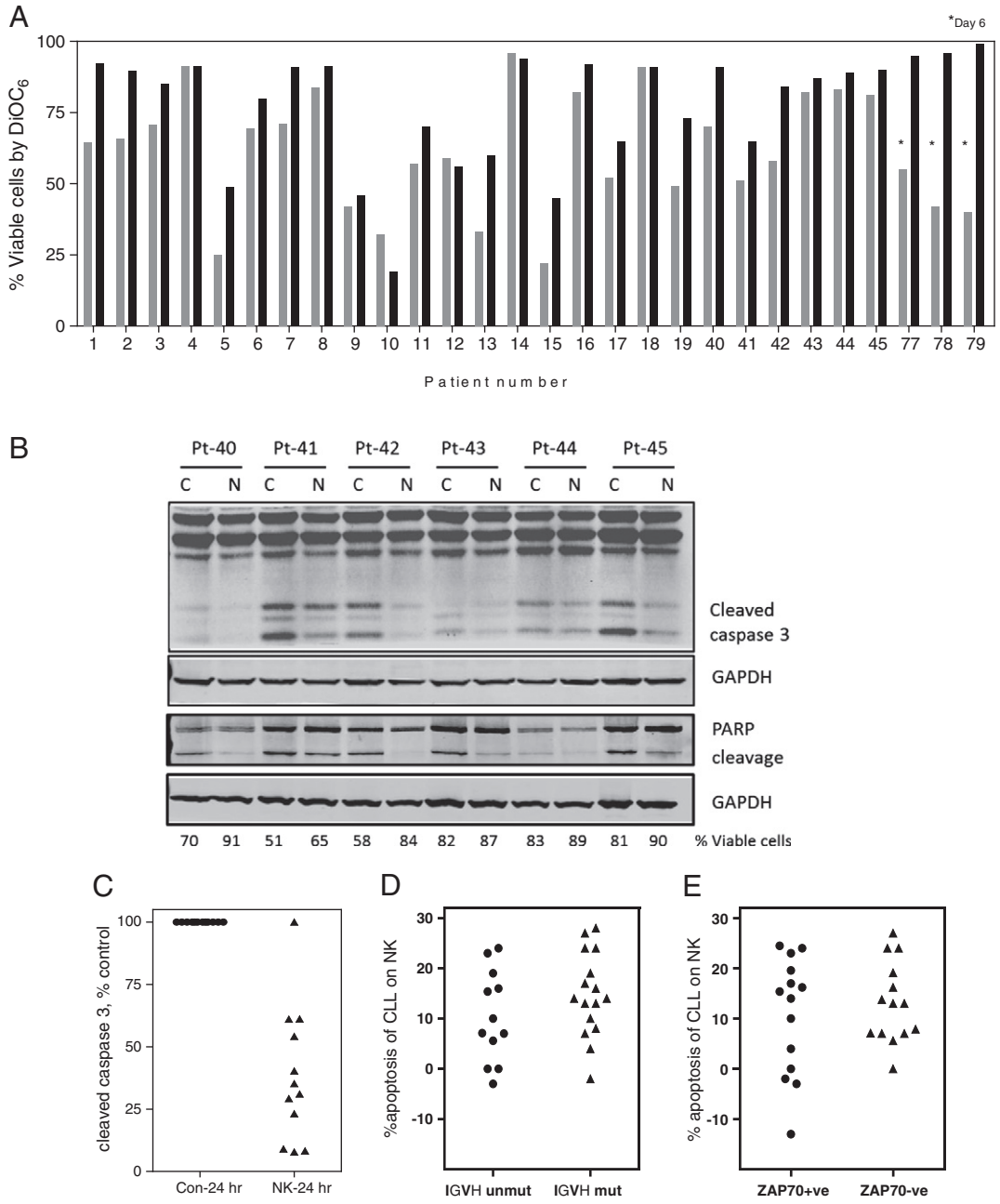


Figure 2. BMSC confers cyto-protection but does not promote proliferation of CLL lymphocytes. A. CLL primary cells are co-cultured either for 24 hours (samples 1 to 19 and 40 to 45) or until day 6 (samples 77 to 79) with or without bone marrow stromal cells (NKtert) in a ratio of 100:1 and the viability of CLL lymphocytes are measured by DiOC6 method (grey bar, CLL cells in suspension; black bar, CLL cells co-cultured on stroma). B. CLL primary cells are co-cultured for 24 hours with or without bone marrow stromal cells (NKtert). Protein extracts are equally loaded and caspase 3 cleavage and PARP cleavage ($n = 6$) are analyzed by immunoblotting (C = CLL in suspension; N = CLL on NKTert). GAPDH is used as a loading control. The percent viable cells for each sample are provided underneath the bands. C. The bands obtained for cleaved caspase-3 (19 kDa) is quantitated, normalized to GAPDH and graphed ($n = 12$). D–E. Stromal cell mediated survival response on patients' samples with different subset of CLL such as I_GV_H mutation and ZAP-70 status. D. Change in percent apoptosis after 24 hours on BMSC is plotted against IgV_H unmutated and mutated samples ($n = 29$). E. Change in percent viability after 24 hours on BMSC in ZAP70-positive and -negative samples ($n = 28$). Twenty-two cases from Figure 1*A* were analyzed for correlation on percent viability of CLL cells with or without stroma (F). CLL primary cells from four patient samples are cultured in presence or absence of BMSC support (NKTert cell line) for extended time (until day 6) and the end points with respect to cell viability (G) and proliferation (H) are measured by annexin/PI and Ki-67 staining methods ($n = 4$). Left panels are quadrant plots of CLL cells cultured for 6 days without stromal support, while right panels are the quadrant plots of CLL cells cultured for 6 days with stromal cells stained with annexin and propidium iodide (G) or Ki-67 and propidium iodide (H).

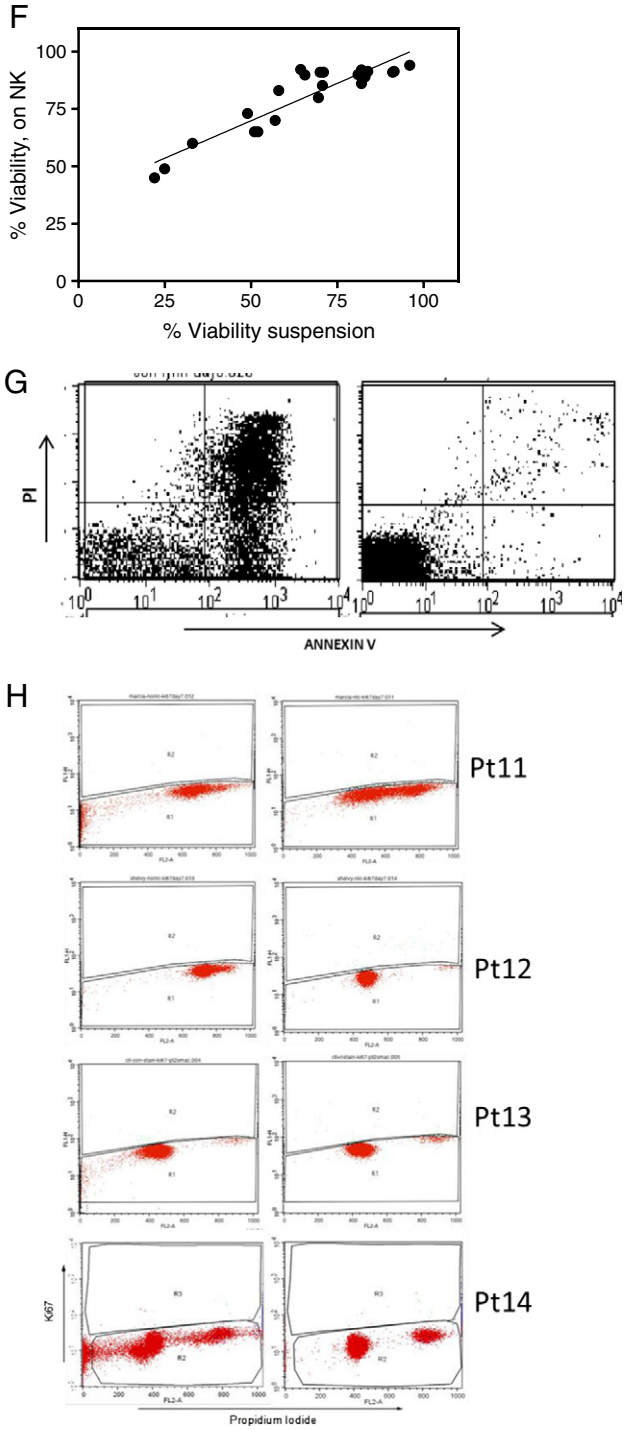


Figure 2 (*continued*).

Measurement of Cell Viability

CLL cell viability is analyzed as described previously by standard methods: annexin V/propidium iodide (PI) binding and 3,3-dihexyloxocarbocyanine iodide (DiOC6) staining, based on the analysis of mitochondrial transmembrane potential and cell membrane permeability to PI [8].

Cell Proliferation Assay

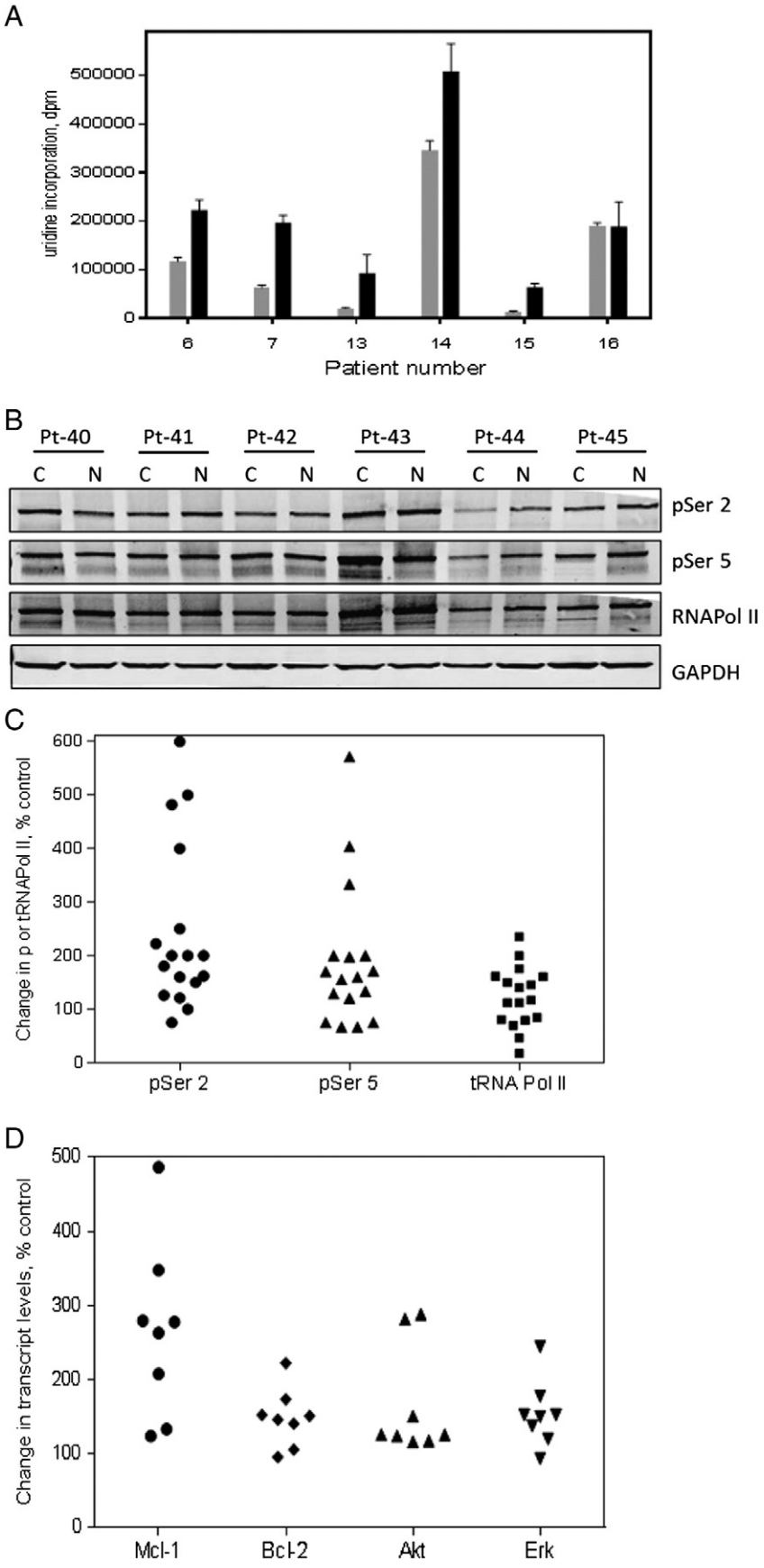
Proliferation is measured using the Ki-67 staining method. Briefly, after incubation, cells are washed with PBS and pelleted at 500 g for 5 min; the pellet is resuspended in 75% ethanol and left on ice. After 30 min, the cells are washed twice and incubated with 20 µl Ki-67 antibody (FITC-conjugated anti-human Ki-67 from BD Biosciences) and incubated in the dark for 30 min, washed with 1% BSA/PBS, added to 10 µl propidium iodide (50 µg/ml), and analyzed immediately with a FACSCALIBUR cytometer (Becton-Dickinson).

Measurement of Global RNA Synthesis

Primary CLL cells in suspension or MSC co-culture are measured for global RNA syntheses by [^{3}H]uridine incorporation assay as described previously [8].

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction

Total cellular RNA is extracted from CLL cells in suspension or in co-cultures using Qiacube (QIAGEN, Valencia, CA). The RNA content and purity is measured by NanoDrop 1000 Spectrophotometer (Thermo scientific Inc, Wilmington, DE). Total RNA (20–50 ng) is used for the one-step real-time reverse-transcriptase polymerase chain reaction (RT-PCR) in the TaqMan One-Step RT-PCR Master Mix



(Applied Biosystems, Foster City, CA). Each PCR reaction is carried out in a 25-μl volume on 96-well optical reaction plate for 30 minutes at 48°C for reverse transcription reaction, followed by 10 minutes at 95°C for initial denaturing, then followed by 40 cycles of 95°C for 15 seconds and 60°C for 2 minutes in the 7900HT Sequence Detection System (Applied Biosystems). The relative gene expression is analyzed by the Comparative Ct method using 18s ribosomal RNA as endogenous control, after confirming that the efficiencies of the target and the endogenous control amplifications are approximately equal. All the primers and probes (*MCL-1*, *BCL-2*, *AKT*, *ERK*) and reverse-transcribed PCR buffers are purchased from Applied Biosystems, with reference ID HS00172036_M1, HS00608023_M1, Hs00178289_m1, Hs01052196_m1, respectively.

Immunoblot Analysis

CLL cell pellets are washed with PBS, lysed on ice in lysis buffer. After protein content is measured using a DC protein assay kit (Bio-Rad Laboratories), the samples are loaded, run and transferred to nitrocellulose membranes (GE Osmonics Labstore) as described previously [9]. Membranes are blocked for 1 hour in blocking buffer and then incubated overnight at 4°C against the following: Akt, phospho-Akt (Ser473), Erk, phospho-Erk, phospho-Gsk-3β (Ser9), phospho-Mcl-1 (Ser159/Thr163) (Cell Signaling Technologies), or for 2 hours at room temperature for Rabbit polyclonal antibody to Mcl-1, Bcl-XL and mouse monoclonal antibody to Bcl-2 (Santa Cruz, CA), actin (Sigma, St. Louis, MO) and GAPDH (Abcam). The antibody to poly (ADP-ribose) polymerase (PARP) is from BIOMOL International (Plymouth Meeting, PA). Antibodies for total RNA pol II (8WG16) and phosphorylated CTD at Ser2 (H5) or Ser5 (H14) are purchased from Covance Research Products (Emeryville, CA). Total GSK-3β antibody (BD biosciences), Bcl2A1 (Epitomics 1639-1), Bcl-xS (Santa Cruz – sc-634, detects both Bcl-xL and xS), BAX (sc20067), BAK (sc7973), BID (CST 2002), BIM (BD Biosciences) and PUMA (CST 4976) are used. After washing with PBS–Tween-20, membranes are incubated with infrared-labeled secondary antibodies (LI-COR Inc) for 1 hour, scanned and visualized using LI-COR Odyssey Infrared Imager.

Statistical Analysis

Linear regression analysis and Student *t* tests (two tailed) are performed using Prism 6 software (GraphPad Software, Inc, San Diego, CA).

Results

Expression of Bcl-2 Family Proteins in CLL Lymphocytes Versus Normal PBMCs

Overexpression of Bcl-2 family anti-apoptotic proteins is commonly associated with unfavorable prognosis in CLL. We compared the expression of Bcl-2 family pro-survival (Figure 1*A*) and pro-apoptotic proteins (Figure 1*B*) between CLL lymphocytes ($n = 9$; freshly obtained from CLL patients) and normal PBMCs ($n = 7$; freshly obtained from age matched healthy donors). The levels of BCL2-A1, Mcl-1, BCL-2 and BCL-xL are relatively higher in CLL lymphocytes compared to normal PBMCs. In regard to pro-apoptotic proteins, while BAK and t-BID expression are comparatively higher in normal PBMCs, Bax levels are similar in both sets of samples. Interestingly, BH3-only pro-apoptotic proteins, BIM and PUMA are higher in CLL lymphocytes. Based on the notion that anti- and pro-apoptotic proteins neutralize each other's function, one would speculate that there will be an inverse correlation between anti-and pro-apoptotic protein levels in a given primary CLL sample. In contrast, there *is* no such association observed between samples suggesting that it is not the total protein; rather its function, regulation and association with other proteins per se may play an important role in disease progression. These data suggests that there is a differential expression of Bcl-2 family proteins between CLL and normal PBMCs.

Given that PBMCs constitutes collection of other cells and CLL cells are majorly B cells, we isolated CD19 + ve B cells from normal PBMCs. The purity of CD19 + ve cells is tested by flow cytometer. The cell extracts from normal B cells ($n = 5$), PBMCs ($n = 3$) and CLL lymphocytes ($n = 4$) are run in parallel. Of note, the expression of Mcl-1, Bcl-2 and Bcl-xl are similar in both normal B cells and CLL cells (Figure 1*C*).

BMSC Confers Cyto-Protection but Does not Promote Proliferation in Quiescent CLL Lymphocytes

Others and we have shown previously that CLL cells co-cultured on MSC enhance CLL survival [9]. Consistent to our previous reports, co-culturing CLL cells on NKTert stromal cells (a human stromal cell line that mimics the bone marrow stroma) increased the percent survival of CLL cells (Figure 2*A*; 24 hour; $n = 25$; $P < .0001$). Extended incubation of CLL cells on stromal cells until day 6 (Figure 2*A*; $n = 3$; denoted by *), reached > 95% viability compared to 45, 58, 60% survival in time-matched controls ($P = .013$). In concert with this data, 17 and 19 kDa cleaved caspase 3 fragments and 85 kDa cleaved PARP fragment that are detected in CLL suspension cultures, significantly diminished in co-cultured samples suggesting that the activation of caspases is reduced by the stromal cells (Figure 2*B*; $n = 6$). Quantitation of immunoblots for cleaved caspase-3 at 19 kDa showed a significant decrease in percent cleavages in cells co-cultured on stroma ($P < .0001$; $n = 12$; Figure 2*C*). Data on caspase 8 and 9 demonstrated similar reduction in activation of these upstream caspases (Supplemental Figure 1). A complete list of patient samples ($n = 45$) and their clinical characteristics are provided in Supplemental Table S1.

There was heterogeneity between samples replenishing on stroma; we therefore investigated if there is any relevant association between patient

Figure 3. MSC-mediated activation of RNA synthesis, RNA pol II protein level and phosphorylation and mRNA transcripts in CLL lymphocytes. CLL lymphocytes ($n = 6$) are incubated in presence (black bar) or absence (grey bar) of BMSC support (NKTert cell line) for 24 hours. A. Global RNA synthesis is measured by uridine incorporation method. The values are presented as absolute DPM in $1*10^7$ cells. The error bars denote mean +/− SEM for the triplicate experiments from each patient samples. B. CLL primary cells ($n = 6$) are co-cultured on stroma for 24 hours and total as well as phosphoRNA polymerase II are analyzed by immunoblotting, using antibodies towards the phosphorylated Ser2 or Ser5 sites of the CTD ofRNA polymerase II. C. Total RNA polymerase II is normalized to GAPDH and phosphorylation at Ser2 and Ser5 of Pol II are normalized to total pol II levels and the immunoblots are quantitated and presented as dot plots. D. CLL lymphocytes are incubated in the presence or absence of MSC (NKTert cell line) for 24 hours and levels of the indicated transcripts are measured by real-time RT-PCR assay. The relative gene expression is analyzed by the comparative Ct method using 18s ribosomal RNA as endogenous control, after confirming that the efficiencies of the target and the endogenous control amplifications are approximately equal. The *P* value was determined by statistical evaluation using two-tailed student paired *t* test.

prognostic markers and *in vitro* cell survival on MSC. The % apoptosis against various prognostic markers is plotted. Number of prior therapies or B_2M levels demonstrated no association with percent viable cells on MSC (data not shown). While apoptosis blockade in IgV_H mutation samples increased slightly compared to IgV_H unmutated samples, it is not statistically significant (Figure 2*D*; unmutated, n = 13, median 8.0% and mutated, n = 16, median 14.0%; P = .1962). Similarly, no difference is observed between ZAP70 positive or negative samples co-cultured on stroma (Figure 2*E*; ZAP70 + ve, n = 14, median 15% and ZAP70 – ve, n = 14, median 13%; P = .5152). No association is seen between percent spontaneous apoptosis and white blood cell count in the peripheral blood (n = 45; data not shown). However, samples with 17p deletion are comparatively resistant to spontaneous apoptosis in culture (data not shown; n = 6). These data suggests that MSC-mediated cyto-protection is independent of prognostic markers and the disease stage. Of note, the increase in percent viability on stroma demonstrated a liner relationship with percent viability with no stroma in the same samples (Figure 2*F*; n = 22; r^2 = 0.84, $P < .0001$).

Previously it was thought that the CLL B-cells are quiescent cells and their accumulation was primarily associated with resistance to apoptosis [23]. However, CLL is currently viewed as a disease characterized by a dynamic balance between quiescent cells circulating in the blood and proliferating cells located in permissive niches in lymphoid organs. To test if MSCs modulate the quiescent nature of CLL cells, lymphocytes co-cultured for longer time on MSCs (up to 6 days) is measured for Ki-67 positivity. Longer incubations with MSCs extended the survival of CLL lymphocytes (Figure 2*G*; day 6), with no indication of replicating populations in the culture, suggesting that MSC does not confer proliferation signals to CLL lymphocytes (Figure 2*H*; day 6; n = 4).

MSC Co-Culturing Activate Total RNA Synthesis in CLL Lymphocytes

CLL cells either cycling or quiescent, require active RNA transcription for the continuous production of housekeeping and other proteins needed for cell survival. Based on this notion, we investigated if the changes in protein levels are indeed due to a direct combinatorial effect of increased transcriptional machinery or by means of indirect mechanisms. To test this, the effect of incubation of CLL cells with NKtert stromal cells on [^{3}H]uridine incorporation is quantified. Data inferred that there is variability in the basal levels of RNA synthesis among samples (grey bars) and the macromolecule synthesis in response to BMSC is heterogeneous (black bars). Variability in the endogenous level of uridine incorporation has been observed before [8,24]. Overall, the rates of RNA synthesis increased significantly indicating that the sustained survival of CLL cells in context to bone marrow microenvironment is in part associated with an increase in global RNA synthesis (Figure 3*A*, n = 6; P = .015).

Activation of Global Transcription Associated With RNA Polymerase II Activity in CLL Cells

Active transcription is dependent on enzyme RNA polymerase II (pol II), as phosphorylation of its carboxyl terminal domain at Ser2 and Ser5 sites is essential for both transcriptional initiation and elongation, respectively. To determine whether increase in rates of RNA synthesis is in conjunction with activation of RNA pol II, the total RNA pol II and the phosphorylation at Ser2 and Ser5 sites are determined. Primary samples co-cultured on stromal cells demonstrated that while total RNA pol II levels remained similar under both culture conditions, there is an increase in phospho RNA pol II at Ser2 (n = 17; P = .007) and Ser5 (n = 17; P = .013) indicating that CLL transcription machinery is activated in association with NK-Tert cells (Figure 3, *B* and *C*).

MSCs Promote the Amplification of mRNA Transcripts of Pro-Survival Molecules in CLL Cells

As the degree of RNA synthesis is in close relationship with percent cell survival, increase in RNA synthesis may be an important biochemical mechanism in stromal-mediated survival of CLL cells. The pro-active global RNA synthesis may eventually amplify functional mRNA transcripts and subsequently increase protein synthesis. Using RT-PCR technique we measured the mRNA transcript levels of *MCL-1*, *BCL-2*, *AKT* and *ERK* kinases that are important for survival of CLL cells. Transcripts for *MCL-1*, *BCL-2*, *AKT* and *ERK* increased on stromal support (P = .006; P = .010; P = .041; and P = .012, respectively); importantly, the greatest and consistent increase in transcript levels is observed for *MCL-1* (Figure 3*D*). Further analysis demonstrated a weak linear correlation between Mcl-1 and BCL-2 (r^2 = .4479; P = .0695) and strong relationship with Erk and Akt (r^2 = 1; P = .0001) transcript levels (data not shown).

Post-Translational Modification of Pro-Survival Molecules in CLL Cells on MSC

Given that cellular protein synthesis depends on functional mRNA transcripts as the templates for translation, it is logical to speculate that increase in RNA synthesis with respect to microenvironment may lead to elevated protein synthesis. We compared the expression of anti-apoptotic proteins (Mcl-1, Bcl-2 and Bcl-xL) in primary cells co-cultured in presence or absence of stroma (Figure 4, *A* and *B*). Among anti-apoptotic proteins, Mcl-1 (n = 48; P = .0006), but not Bcl-2 (n = 34; P = .46) and Bcl-xL expression (n = 16; P = .26; Figure 4*B*) increased in co-cultured cells demonstrating a positive correlation between transcription and translation of short-lived proteins. As PARP cleavage is a direct measure of cell death, we correlated PARP cleavage with Mcl-1 induction in presence of stroma (Figure 4, *C* and *D*; n = 8). There is a very weak not-significant inverse correlation between these two parameters (Figure 4*D*; n = 8; P = .314).

Expression of Protein Kinases in CLL Lymphocytes Versus Normal PBMCs

In order to derive the significance of protein kinases in co-cultured CLL lymphocytes, the expression of Akt and Erk in normal lymphocytes, CLL lymphocytes and cells co-cultured on stroma (Figure 5, *A–D*) are evaluated. Total Erk protein is similar in both CLL and normal lymphocytes. While total Akt levels were undetectable in normal PBMCs, but is expressed in CLL lymphocytes; however, both Akt and Erk phosphorylation increased in CLL cells co-cultured on MSC (Figure 5, *B* and *C*). Quantitation of immunoblots revealed statistical significance for pAkt (P = .04; n = 18) and Erk phosphorylation (P = .037; n = 6; Figure 5*D*).

GSK-3β is a substrate of Akt and Akt induces post-translational modification of GSK-3β leading to loss of function, which otherwise will phosphorylate its cognate substrate Mcl-1 at a conserved GSK-3 phosphorylation site (Ser159) promoting ubiquitination and degradation of Mcl-1. Therefore, the levels of Gsk-3β protein in CLL

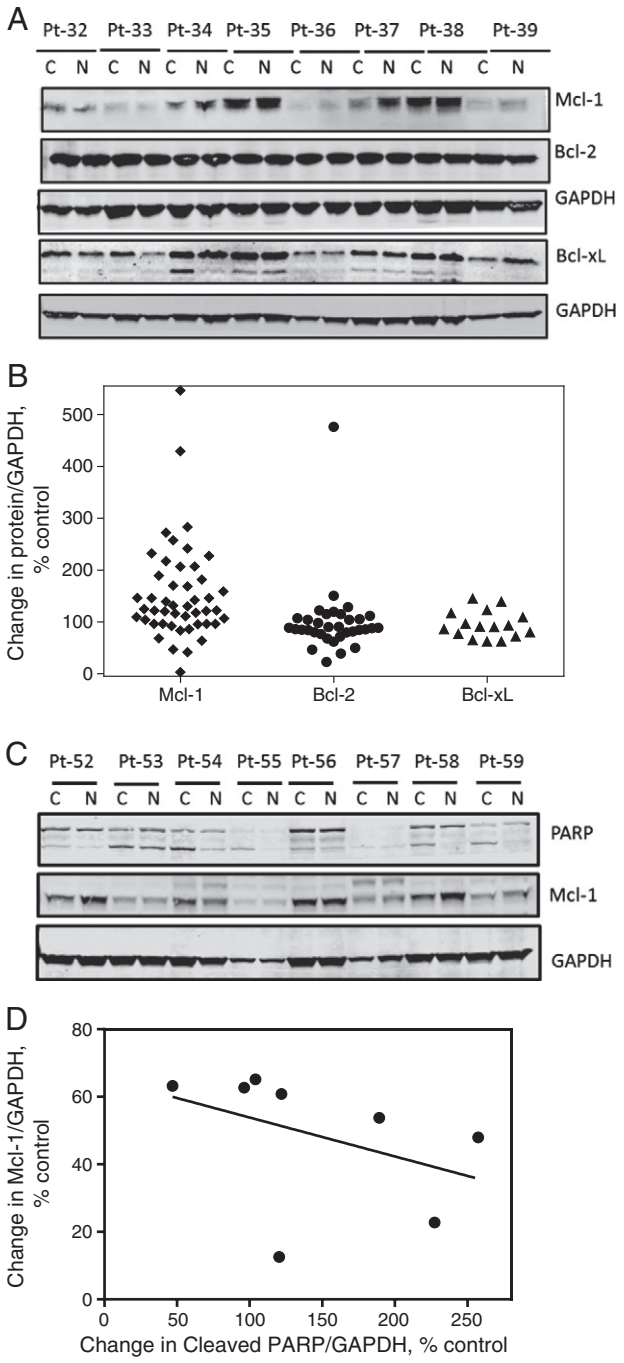


Figure 4. MSC mediated changes in the expression of anti-apoptotic proteins in CLL primary cells. A. CLL primary cells are co-cultured for 24 hours with or without bone marrow stromal cells (NKtert) in a ratio of 100:1 and the protein extracts are equally loaded to detect the anti-apoptotic proteins, Mcl-1, Bcl-2 and Bcl-xL (n = 8) using respective antibodies by immunoblotting analysis (C = CLL in suspension; N = CLL on NKTert). GAPDH is used as a loading control. B. The immunoblots for Mcl-1 (n = 48), Bcl-2 (n = 34) and Bcl-xl (n = 16) are quantified and normalized to the loading control GAPDH. The P values are determined by statistical evaluation using two-tailed student paired t-test. C and D. Correlation between Mcl-1 and PARP cleavage was derived. C. Immunoblots were performed for Mcl-1 and PARP cleavage in the same samples (n = 8). GAPDH is used as a loading control. D. Correlation between Mcl-1 and PARP cleavage is derived using linear regression analysis by Prizm software.

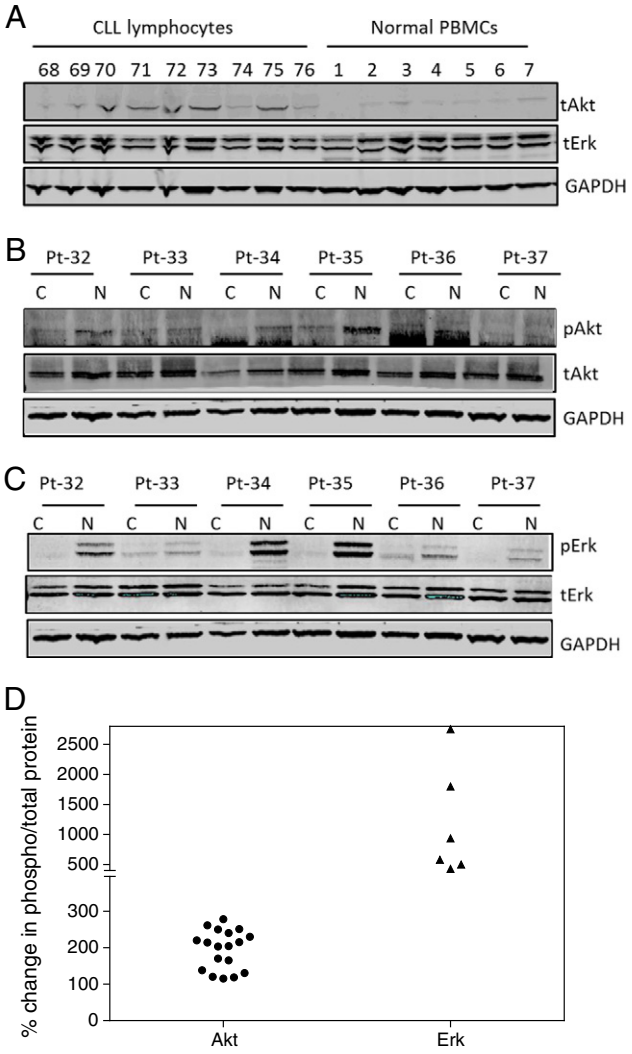


Figure 5. MSC mediated changes in pro-survival kinases in CLL primary cells. A. Protein extracts obtained from CLL lymphocytes (n = 9) and normal lymphocytes (n = 7) are loaded in equal amounts in the same gel and the protein levels of tAkt and tErk are evaluated by immunoblotting analysis. B. CLL primary cells (n = 6) are co-cultured for 24 hours with or without bone marrow stromal cells (NKtert) in a ratio of 100:1 and the protein extracts are equally loaded to detect tAkt and pAkt (Ser473) C. CLL primary cells (n = 6) are co-cultured for 24 hours with or without bone marrow stromal cells (NKtert) in a ratio of 100:1 and the protein extracts are equally loaded to detect tErk and pErk by immunoblotting analysis (C = CLL in suspension; N = CLL on NKTert). GAPDH is used as a loading control. D. The phospho protein immunoblots (Akt and Erk) are quantified and normalized to the total protein for cells on stroma, graphed and compared with the cells in suspension cultures (100% control) and plotted as dot plots.

lymphocytes, normal lymphocytes and cells co-cultured on stroma are measured (Figure 6, *A–C*). Total Gsk-3β is significantly higher in CLL cells compared to normal lymphocytes (Figure 6*A*). Comparison of total Gsk-3β protein and its phosphorylation status (at Ser9) between CLL cells and cells co-cultured on stroma demonstrated that pGsk-3β levels are significantly higher in cells co-cultured on stroma (Figure 6, *B* and *C*; P = .0001; n = 18).

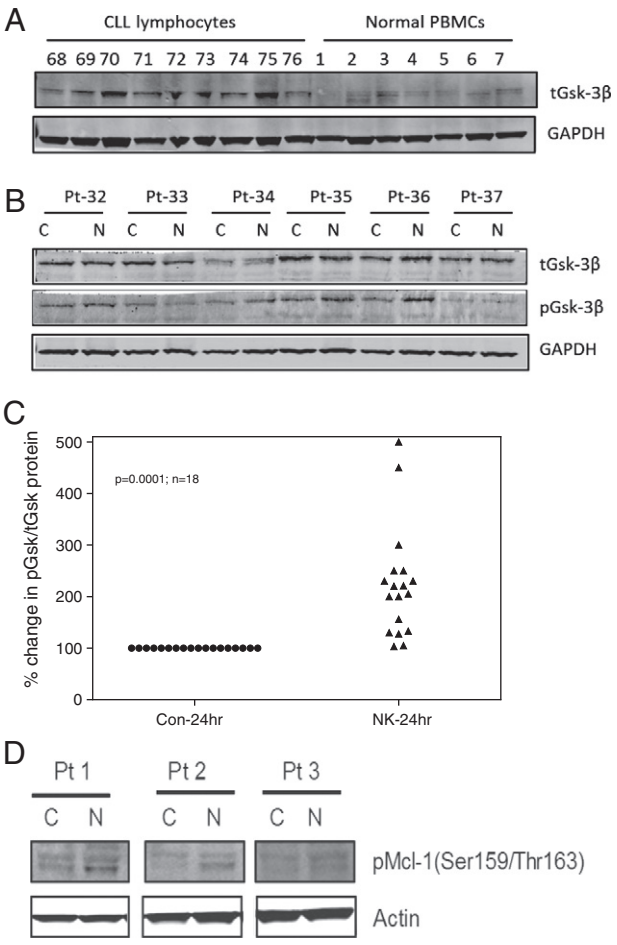


Figure 6. MSC mediated changes in Gsk-3β in CLL primary cells. A. Protein extracts obtained from CLL lymphocytes (n = 9) and normal lymphocytes (n = 7) are loaded in equal amounts side by side and the protein levels of tGSK and pGSK are evaluated by immunoblotting analysis. B. CLL primary cells (n = 6) are co-cultured for 24 hours with or without bone marrow stromal cells (NKtert) in a ratio of 100:1 and the protein extracts are equally loaded to detect tGsk-3β and pGsk-3β (Ser9). C. pGsk-3β immunoblots is quantified and normalized to total protein and plotted as dot plots. D. Mcl-1 phosphorylation at Ser159/Thr163 is measured by immunoblotting analysis.

There is a steady-state equilibrium between the production and elimination of these molecules, the post-translational modifications of proteins (as reviewed previously [25]) may further stabilize their expression and function. In conjunction to this hypothesis, there is an increase in post-translational modification of protein Mcl-1 at Ser159/Thr163 (Figure 6*D*) on co-cultured cells leading to Mcl-1 stabilization.

Discussion

Mcl-1 protein is a vital component of survival pathway and its regulation of expression and function are critical in cell survival [1,10,26]. It has been previously shown that out of six members of the Bcl-2 family anti-apoptotic proteins, Mcl-1 is up-regulated in CLL cells in BMSC milieu [9,10], however the mechanism by which it is regulated is not known. Given that DNA synthesis is not an active episode in quiescent CLL, transcription is an essential process in CLL cells. In the same line, treatment with RNA directed agents promoted cytotoxicity in CLL cells [27] and inhibition of mRNA synthesis has been shown effective in killing CLL cells, suggesting that transcription could be a principal target in quiescent cells [28]. Proof of concept of this hypothesis was demonstrated by CDK7 and CDK9 inhibitors such as roscovitin, flavopiridol [4,29], and SNS-032 [30,31] that showed greater cytotoxicity towards CLL cells both in pre-clinical models and clinical trials [31,32]. Importantly, the mechanism by which CDK inhibitors induced cell death is through the blockade of transcriptional events leading to a decline in mRNA and protein levels of anti-apoptotic protein Mcl-1. As transcription is proven a potential target in CLL, the resistance to apoptosis of CLL cells on MSC is mediated through activation of transcriptional machinery suggesting that the survival mechanisms promoted by MSCs is likely dependent upon transcription that is regulated by both intrinsic molecules and extrinsic signals derived from the microenvironment [8].

Mcl-1 is a protein that is inherently programmed for fast turn-over [33] due to PEST sequences in the protein. However, Erk-mediated phosphorylation of Thr163 in the PEST domain diminished the degradation rate of Mcl-1 protein in Burkitt's lymphoma B-cell line [34]. Consistently, inhibition of Erk pathway by sorafenib is effective in inducing apoptosis in primary CLL lymphocytes [35]. Post-translational modifications of Mcl-1 protein at Ser121 residue leading to its inactivation has been reported in HEK293, however this modification *is* in response to stress [36]. Similarly, phosphorylation of Ser64 site of Mcl-1 protein by CDKs has been demonstrated to be associated with enhancement of anti-apoptotic property of the protein [37]. Additionally, Mcl-1 protein is modified to undergo proteasomal degradation after ubiquitylation by an E3 ligase, MULE [38]. Another novel E3 ligase that is selective for Mcl-1 is recently reported to regulate degradation of this anti-apoptotic protein [39]. On the other hand, degradation of Mcl-1 is also regulated by deubiquitination (DUB), USP9X [40] a specific enzyme for Mcl-1 deubiquitination revealing that stability of Mcl-1 is balanced through confined post-translational modifications and concurrent proteasomal degradation pathways.

Gsk-3β (glycogen synthase kinase 3) controls Mcl-1 stability and regulation of apoptosis [41]. Several kinases are known to mediate post translational modification of Gsk-3β among which Akt is one of its known kinases [42,43]. In presence of PI3K/Akt signaling axis, Akt induces post-translational modification of Gsk-3β, which phosphorylates its cognate substrate Mcl-1 at a conserved GSK-3 phosphorylation site (Ser159) promoting its ubiquitination and degradation. Thus, Mcl-1 is a substrate of Gsk-3β and the expression of Mcl-1 is correlated with pGsk-3β (Ser9) in multiple cancer cell lines and primary samples [44,45]. Mechanistic studies have suggested that Akt mediated Gsk-3β inactivation plays a role in Mcl-1 phosphorylation and stabilization [26,46]. Activation of GSK-3β results in Mcl-1 degradation, while inactivation of GSK-3β causes accumulation of Mcl-1 promoting chemoresistance in a Mcl-1 dependent manner. In line, when CLL cells are co-cultured on stroma, there is a consistent occurrence of increase in phosphorylation of GSK-3β (glycogen synthase kinase 3; Figure 6) and concurrent Mcl-1 accumulation. While rate of degradation was not determined in our studies, Erk activation, phosphorylation of Mcl-1 at Thr163 and concurrent accumulation of Mcl-1 are observed in CLL-MSC co-cultures (Figures 5 and 6).

On the basis of these observations, we present a molecular model (Figure 7) that illustrates the regulation of expression and modifications of Mcl-1 with respect to MSC. The stromal-mediated increase in RNA synthesis could be due to activation of transcription factors such as *c-MYC*

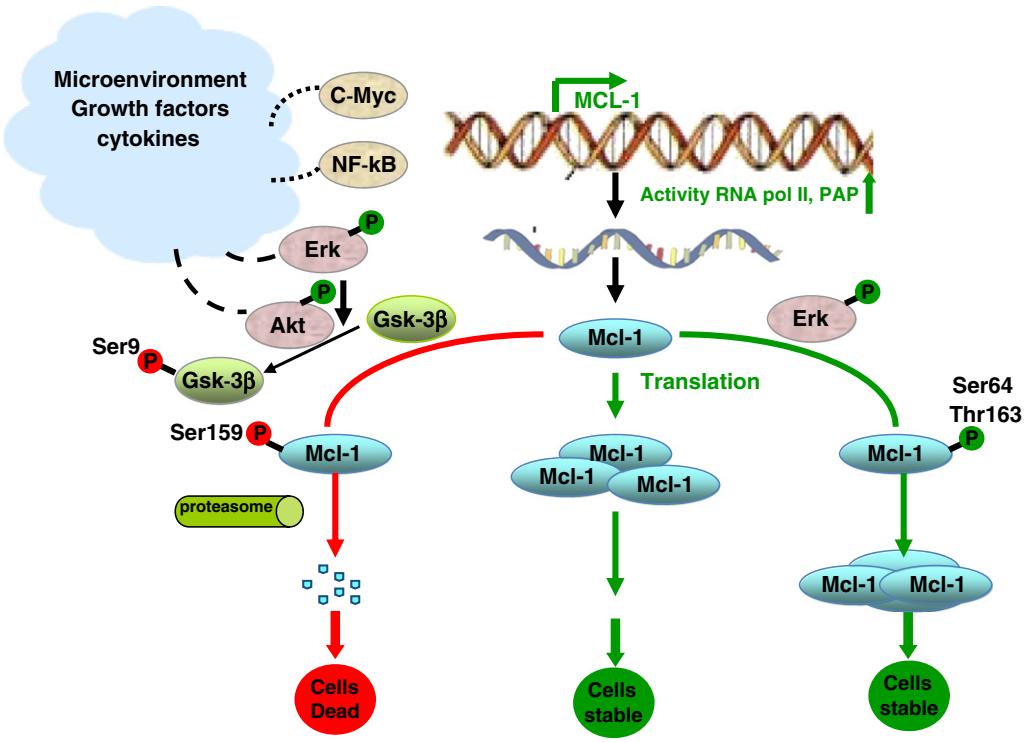


Figure 7. Molecular model of Mcl-1 regulation in CLL lymphocytes by bone marrow stromal microenvironment. Model representing the regulation of Mcl-1 at various checkpoints such as transcriptional, translational and post-translational modifications in CLL cells with respect to MSCs.

and NF-κB. Increase in transcription concurrently amplifies mRNA production and subsequently elevates protein synthesis. Early response genes such as Mcl-1 are induced to a much greater extent than the RNA transcripts with slow turnover rates. If the induction of short-lived transcripts and proteins is thought to be a biochemical mechanism for the survival of CLL cells, one would expect that direct inhibition of transcription and translation by respective inhibitors may have a cytotoxic effect on CLL cells; consistently, transcriptional and translational inhibitors have demonstrated promising therapeutic strategies for CLL [34,47,48].

Additionally, Mcl-1 is regulated by BCR signaling kinases such as Akt, Erk and GSK. Phosphorylation of GSK-3β by Akt inactivates GSK-3β and concurrent Mcl-1 degradation [41]. On the other hand, Erk-mediated Mcl-1 phosphorylation at Thr163 in the PEST region stabilizes this anti-apoptotic protein [34]. Harmonious with these data, inhibition of PI3K delta by Cal-101 or SYK by R408 or Erk by sorafenib resulted in malignant B-cell apoptosis in vitro [46] and during therapy [49] in primary CLL cells [19,37,50].

In conclusion, the dynamic equilibrium between the stabilization and degradation of Mcl-1 dictates the fate of a malignant cell. Because Mcl-1 is a short-lived transcript and protein with high turnover rate [51], any modulation with respect to cellular response would significantly impact its alteration and expression. Our data demonstrate a concerted effect on Mcl-1 in CLL cells influenced by bone marrow microenvironment.

Disclosure of Conflict of Interest

The authors have no financial conflict of interests.

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