# Synergistic effect of *Bcl2*, *Myc* and *Ccnd1* transforms mouse primary B cells into malignant cells

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## ABSTRACT

### Background
A synergistic effect resulting from a combination of *BCL2* and *MYC* or *MYC* and *CCND1* has been implicated in human B-cell lymphomas. Although the identification of other cooperative genes involved is important, our present understanding of such genes remains scant. The objective of this study was to identify the additional cooperative gene(s) associated with *BCL2* and *MYC* or *MYC* and *CCND1*. First, we assessed whether *Bcl2*, *Myc* and *Ccnd1* could cooperate. Next, we developed a synergism-based functional screening method for the identification of other oncogene(s) that act with *Bcl2* and *Myc*.

### Design and Methods
Growth in culture, colony formation and oncogenicity *in vivo* were assessed in mouse primary B cells exogenously expressing various combinations of *Bcl2*, *Myc* and *Ccnd1*. For the functional screening, *Bcl2*- and *Myc*-expressing primary B cells were infected with a retroviral cDNA library. Inserted cDNA of transformed cells in culture were then identified.

### Results
Primary B cells exogenously expressing *Bcl2*, *Myc* and *Ccnd1* showed factor-independent growth ability, enhanced colony-forming capability and aggressive oncogenicity, unlike the cases observed with the expression of any combination of only two of the genes. We identified *CCND3* and *NRAS* as cooperative genes with *Bcl2* and *Myc* through the functional screening.

### Conclusions
*Bcl2*, *Myc* and *Ccnd1* or *Bcl2*, *Myc* and *CCND3* synergistically transformed mouse primary B cells into aggressive malignant cells. Our new synergism-based method is useful for the identification of synergistic gene combinations in tumor development, and may expand our systemic understanding of a wide range of cancer-causing elements.

Key words: cooperative genes, malignant transformation, oncogenes, *Bcl2*, *Myc*, *Ccnd1*.

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*The online version of this article has a Supplementary Appendix.*

## Introduction

Intense efforts to examine genetic alterations in human cancer have provided a catalogue of cancer-causing genes. It is speculated that most cancers arise as a result of accumulation of altered cancer-causing genes. The study of functional synergism among cancer-causing genes is thus becoming one of the central issues in cancer research.[1] However, it is difficult to determine which combinations of genes are involved in oncogenesis because of the large number of possible candidates. This accounts for the fact that our knowledge about synergistic combinations of cancer-causing genes has remained limited.

Genetic alterations of *BCL2* (B-cell CLL/lymphoma 2), *MYC* [v-myc myelocytomatosis viral oncogene homolog (avian)] and *CCND1* (cyclin D1) are the most frequently found alterations in human B-cell lymphomas. These genes are transcriptionally deregulated as the partner genes of *IgH* translocation, and are thus thought to perform crucial roles in human B-cell lymphomagenesis.[2] A synergistic effect resulting from a combination of two genes from *BCL2*, *MYC* and *CCND1* has been implicated in human B-cell lymphomagenesis. About 4% of cases of diffuse large B-cell cell lymphoma possess *BCL2/MYC* double translocations, suggesting a synergistic effect of *BCL2* and *MYC* in lymphoma development.[3-7] The synergistic effect of *BCL2* and *MYC* has also been implicated in the histological and clinical transformation of indolent follicular lymphoma into a more aggressive lymphoma.[8-12] Cases of *CCND1/MYC* double translocation are relatively frequent in mantle cell lymphoma.[7] Importantly, it is believed that other hitherto unknown genes also play important roles in lymphomagenesis in addition to the synergistic effects of the aforementioned two oncogenes since human B-cell lymphomas often show a variety of genes subject to alterations and/or deregulated expression.[7] That multiple genes are involved in human lymphoma formation has also been suggested by experimental mouse models. Cory *et al.* pointed out that additional genetic alterations were involved in lymphoma development in an Emu mouse model ectopically expressing *Myc* and *Bcl2*.[13] The identification and clarification of the multiple cooperative genes implicated in human lymphoma formation is important, although our present understanding of such genes remains scant.

The objective of this study was to identify additional cooperative gene(s) associated with *BCL2* and *MYC* or *MYC* and *CCND1* in human B-cell lymphomagenesis. Importantly, human B-cell lymphoma cases with concurrent multiple translocations including *BCL2*, *MYC* and *CCND1* have been reported.[14,15] Given the different biological functions of *BCL2*, *MYC* and *CCND1*,[16-20] we investigated the possibility that these three genes might act synergistically. *In vitro* assays demonstrated that ectopic expression of all three genes could transform mouse primary B-cells, unlike the cases observed following the expression of any combination of only two of the three genes. We also determined that this synergistic effect contributed to lethal tumor development in mice. Furthermore, we used these findings to develop a new functional screening method, with which we were able to identify other transforming gene combinations.

## Design and Methods

### Generation of retrovirus

Retroviral vector plasmids were transiently co-transfected with MCV-Ecopac vector[21] (kindly provided by Dr. Richard Van Etten, Tufts-NEMC Cancer Center, Boston, MA, USA) into 293 T cells using the calcium phosphate precipitation method (Profection mammalian transfection system; Promega) or the FuGENE6 transfection reagent (Roche) according to the manufacturers' instructions. Twenty-four hours following transfection, the culture medium was replaced with Feeder medium [Iscove's modified Dulbecco's medium (IMDM) supplemented with 2% fetal calf serum (FCS) containing 2- mercaptoethanol ($5\times10^{-5}$ M; Sigma, St. Louis, MO, USA) and Primatone RL (0.03% wt/vol; Cellgro) with interleukin-7 [IL-7; 5% of mouse IL-7-producing cell line J558 supernatant (kindly provided by Dr. Tariq Enver, University of Oxford, Oxford, UK)]. Cells were incubated at 32°C for 24 h before harvesting of the virus supernatants. The virus supernatants were filtered (0.45 μM) and then frozen at -80°C.

### Preparation and retroviral infection of cells

On day 15 or 16 of gestation, fetal liver cells were enriched to produce pro B-cells by cell sorting for B220 and c-Kit expression, and cultured at $5\times10^{3}$ /mL in Feeder medium containing IL-7 (5%) on 15 Gy-irradiated ST-2 stromal cells. Following pro B-cell growth for 3-4 days, cells were used for retroviral infection. Cells ($1.25\times10^{5}$) were plated onto ST-2 cells in a 6-well culture plate (Costar). After 24 h, cells were suspended in virus supernatant and spin infected at 2000 rpm (840g, 1.5 h) at room temperature. For drug selection, cells were serially infected with various retroviral vectors and then purified by serial selection using neomycin (1.2-2 mg/mL), hygromycin (0.3-1.5 mg/mL) and blasticidin (15-20 μg/mL). For cell sorting, cells were simultaneously infected with various retroviral vectors and sorted for green fluorescent protein (GFP) and huKO expression after 2 or 3 days. In Screening 2, cells were infected with MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP* and sorted for GFP expression. The combination of retroviral vectors used for establishing the various stable integrants are detailed in *Online Supplementary Figure S1*.

### In vitro *liquid assays*

Prior to commencement of the *in vitro* liquid assay without IL-7 and ST-2 cells, cell samples were washed twice in Feeder medium. Cells ($1\times10^{5}$ or $5\times10^{5}$) were seeded into wells of a 24-well plate on day 0.

### In vitro *colony-forming assays*

Cells were plated in triplicate onto 35-mm dishes in 1 mL of semisolid, 1% methylcellulose-based medium supplemented with FCS (30%), mouse stem cell factor (SCF, 5% of mouse SCF producing a cell line supernatant), mouse IL-7 (5%), and mouse FLT3 (FMS-like tyrosine kinase 3) ligand (10 ng/mL; 250-31L, Peprotech)

### In vivo *transplantation assays*

Cells ($1\times10^{7}$) were transplanted intravenously into SCID mice. Cell suspensions of spleen or lymph nodes were obtained by pressing the tissue gently between two glass microscope slides and then treating the sample with ACK (0.15 M NH4Cl, 1.0 mM KHCO3 and 0.1 mM EDTA) to lyse the red blood cells. Tissues were fixed in phosphate-buffered saline containing 10% formaldehyde, embedded in paraffin, and then stained with hematoxylin and eosin. Experiments were performed with the approval of the Institutional Ethical Committee for Animal Experiments of Aichi Cancer Center Research Institute.

### *Construction of the retroviral cDNA expression library and screening*

Construction of the retroviral cDNA expression library was performed as previously described.[22] In brief, a double-stranded cDNA longer than 1.0 kb obtained from the SU-DHL-6 cell line was ligated into a *Bst*XI digested pMXs vector. These procedures allowed the use of an endogenous stop codon of each gene in this expression library system. *Escherichia coli* DH10B was transformed by electroporation, resulting in the production of about $1.0\times10^6$ clones. To obtain the results from functional screenings, the inserted cDNA were recovered by PCR-amplification using the retroviral-specific primers (S: 5'-GGTGGACCATCCTCTAGACT-3', AS: 5'-CCCTTTTTCTGGAGACTAAAT-3') from the retrovirus-integrated genomic DNA of proliferating cells. Anti-sense oriented cDNA clones were excluded from analysis in this study.

### *Statistical analysis*

Mean values ± s.e.m. were used throughout. Data were analyzed using the Student's t-test and Kaplan–Meier survival analysis was conducted using Statview software (SAS Institute).

Further details on the construction of vectors, flow cytometry, Southern blot analysis and analysis of expression microarray data are provided in the *Online Supplementary Design and Methods*.

## Results

### In vitro *growth of primary B cells with deregulated expression of* IgH *translocation-associated oncogenes*

A defining characteristic of the transformed cell *in vitro* is the lack of dependency on exogenous proliferating and/or survival signals. As BALB/c fetal liver-derived pro B-cells could be cultured for over 6 months in the presence of IL-7 on ST-2 stromal cells,[23,24] our initial efforts focused on identifying oncogenes that impart B cells independence from IL-7 and ST-2 cells. We infected primary B cells with retrovirus containing one oncogene (*Bcl2*, *Myc* or *Ccnd1*) and a drug selection marker. Drug-selected cells with a single gene could not proliferate in the absence of IL-7 and ST-2 cells (*data not shown*). All combinations comprising two genes from *Bcl2*, *Myc* and *Ccnd1* were also unable to transform primary B cells (Figure 1A, Experiment (EXP) 1, see also *Online Supplementary Figure S1* for vector constructions and stable integrants). In marked contrast, primary B cells stably expressing *Bcl2*, *Myc* and *Ccnd1* (*Online Supplementary Figure S2A*) showed stable growth in the absence of IL-7 and ST-2 cells (Figure 1A, EXP 1). These findings were reproducible (Figure 1A, EXP 2).

To confirm these initial results, primary B cells stably expressing exogenous *Bcl2*, *Myc* and *Ccnd1* were established with a non-drug selection method. We simultaneously infected primary B cells with two vectors. One vector co-expressed *Bcl2*, *Myc* and *GFP* (MSCV-*Bcl2*-pgk-*Myc*-ires-*GFP*)[25] and the other vector co-expressed *Ccnd1* and *humanized Kusabira-Orange* (*huKO*)[26] (pGCDNsam-*Ccnd1*-ires-*huKO*) (*Online Supplementary Figure S1*). GFP and huKO double-positive cells were purified by cell sorting and designated as "Bcl2/Myc/Ccnd1". Ectopic expression of BCL2, MYC and CCND1 proteins in Bcl2/Myc/Ccnd1 cells was confirmed by western blot analysis (*Online Supplementary Figure S3*). This procedure allows for faster purification of the stable integrant compared to the drug selection method, and hence minimizes uncontrollable factors that may have additive effects on cellular transformation. Bcl2/Myc/Ccnd1 cells could grow in the absence of IL-7 and ST-2 cells, unlike Bcl2/Myc/mock cells (Figure 1B, EXP 1, see also *Online Supplementary Figure S1* for vector constructions and stable integrants). Other stable integrants stably expressing all three genes ("Ccnd1/Myc/Bcl2" and "Bcl2/Ccnd1/Myc") were also established (see *Online Supplementary Figure S1* for vector constructions and stable integrants and *Online Supplementary Figure S3* for protein expression). As expected, both Ccnd1/Myc/Bcl2 and Bcl2/Ccnd1/Myc cells could grow in the absence of IL-7 and ST-2 cells, unlike Ccnd1/Myc/mock and Bcl2/Ccnd1/mock cells (Figure 1B, EXP 1). Reproducibility was demonstrated by an independent experiment (Figure 1B, EXP 2). These results confirmed that ectopic expression of *Bcl2*, *Myc* and *Ccnd1* could synergistically transform primary B cells to gain independence from IL-7 and ST-2 cells.

### *Enhanced colony-forming efficiency of primary B cells with deregulated expression of* Bcl2, Myc *and* Ccnd1 *in a methylcellulose matrix*

The ability to form colonies in semisolid media is another characteristic feature of transformed cells. As drug-selected stable integrants expressing the three genes formed more colonies than those expressing any combination comprising only two of the genes (*Online Supplementary Figures S2B,C and S4*), we confirmed these results using sorted stable integrants. In the first plating, Bcl2/Myc/Ccnd1, Ccnd1/Myc/Bcl2 and Bcl2/Ccnd1/Myc cells showed statistically significant more efficient colony formation than Bcl2/Myc/mock, Ccnd1/Myc/mock and Bcl2/Ccnd1/mock, respectively (Figure 1C, EXP 1, and D). In the re-plating, cells expressing any combination of the two genes did not show any colonies, while Bcl2/Myc/Ccnd1, Ccnd1/Myc/Bcl2 and Bcl2/Ccnd1/Myc cells formed a substantial number of colonies (Figure 1C, EXP 1). Similar results were obtained using an independent experiment (Figure 1C, EXP 2). These results indicate that the ectopic expression of *Bcl2*, *Myc* and *Ccnd1* synergistically transforms primary B cells so that they acquire efficient colony-forming capability in a methylcellulose matrix. However, the colony-forming efficiency of the stable integrants with *Bcl2*, *Myc* and *Ccnd1* was much lower than that of the human B-cell lymphoma cell lines SU-DHL-6 and Raji (Bcl2/Myc/Ccnd1 $25/10^5$ (0.025%); Ccnd1/Myc/Bcl2 $5/10^5$ (0.005%); Bcl2/Ccnd1/Myc $141/10^5$ (0.141%); SU-DHL-6 $21/10^3$ (2.1%); Raji $864/10^3$ (86.4%) in the first plating).

### *The contribution of deregulated expression of* Bcl2, Myc *and* Ccnd1 *in converting primary B cells into highly aggressive malignant cells*

In an effort to examine whether the synergistic effect of *Bcl2*, *Myc* and *Ccnd1* could contribute to tumor development, we transplanted $10^7$ cells of the sorted stable integrants into non-irradiated SCID mice. SCID mice transplanted with Bcl2/Myc/Ccnd1, Ccnd1/Myc/Bcl2 or Bcl2/Ccnd1/Myc cells developed fatal leukemia/lymphoma more rapidly than mice transplanted with cells containing only two of the oncogenes [Figure 2A, EXP 1, average survival time: 63.5 days for the cells expressing three genes (Bcl2/Myc/Ccnd1, n=6; Ccnd1/Myc/Bcl2, n=6; and Bcl2/Ccnd1/Myc, n=6) *versus* 163.4 days for cells expressing two genes (Bcl2/Myc/mock, n=3; Ccnd1/Myc/mock, n=2 and Bcl2/Ccnd1/mock, n=3),

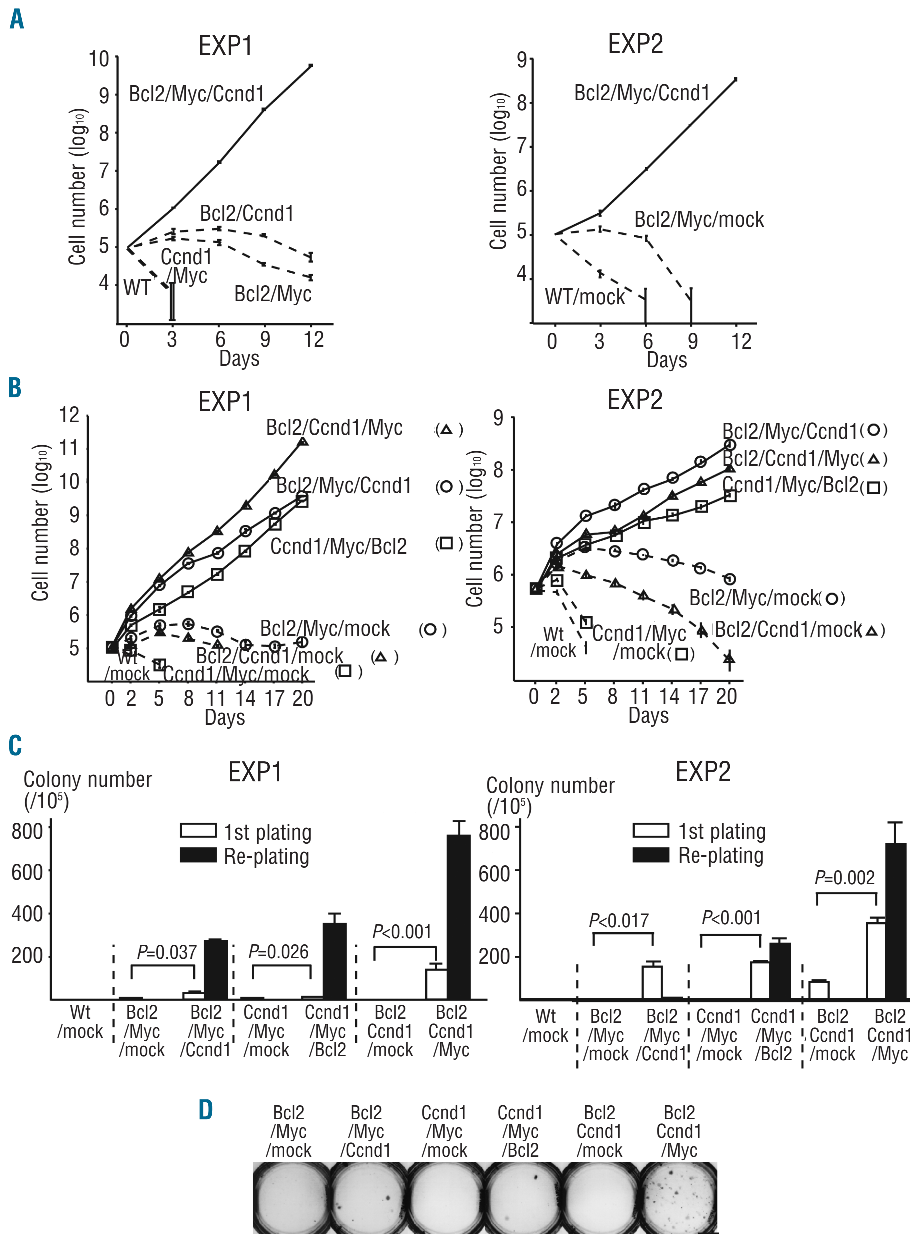


**Figure 1.** Transformation of primary mouse B cells stably expressing exogenous *Bcl2*, *Myc* and *Ccnd1 in vitro*. (A and B) *In vitro* growth curves of various stable integrants established by the drug selection (A) or cell sorting (B) methods in the absence of IL-7 and ST-2 cells. Right and left panels show the results of Experiment 1 (EXP 1) and Experiment 2 (EXP 2), respectively. Data are the mean ± s.e.m. (triplicate). (C and D) Methylcellulose colony-forming abilities of various stable integrants established by the cell sorting method (C) and photograph of representative colonies in the first plating (D). Right and left panels in (C) show the results of EXP 1 and EXP 2, respectively. Data are the mean±s.e.m. (triplicate). *P* values are two-sided (Student's test). Scale bar represent 10 mm.

*P*=0.0003; Figure 2B, EXP 1, average survival time: 51.3 days for Bcl2/Myc/Ccnd1 *versus* 83.7 days for Bcl2/Myc/mock, *P*=0.0128; Figure 2C, EXP 1, 97.0 days for Ccnd1/Myc/Bcl2 *versus* 243.5 days for Ccnd1/Myc/mock, *P*=0.0319; Figure 2D, EXP 1, 42.2 days for Bcl2/Ccnd1/Myc *versus* 189.7 days for Bcl2/Ccnd1/mock, *P*=0.0051]. Dissections of two mice with Bcl2/Myc/Ccnd1 and four mice with Bcl2/Ccnd1/Myc showed that $GFP^{+}huKO^{+}$ tumor cells had mainly infiltrated the enlarged lymph nodes, spleen and thymus (Figure 2E,F), and bone marrow was also saturated with tumor cells. Fluorescence-activated cell sorter (FACS) analysis of the surface markers of the malignant cells infiltrating lymph nodes indicated that they had an immature B-cell phenotype (pre B; c-Kit$^{-}$, B220$^{+}$, CD19$^{+}$, IgM$^{-}$). Southern blot analysis of genomic DNA extracted from enlarged lymph nodes showed that all cases comprised one to several clonal *IgH* rearrangement(s) and that the patterns of *IgH* rearrangements among these cases differed (Figure 2G, lanes 4,5,7-10). Reproducibility was determined using an independent experiment (Figure 2A-D, EXP 2). Consistent results were also obtained when drug-selected stable integrants were transplanted into SCID mice (Figure 2H). All of these findings obtained with our *in vivo* mouse model clearly indicate that ectopic expression of *Bcl2*, *Myc* and *Ccnd1* can transform primary B cells so that they develop a highly aggressive malignant potential.

### *Screening a retroviral cDNA expression library to identify functional genes cooperating with* Bcl2 *and* Myc *in human B-cell lymphoma*

Having established that the synergistic effect of the *IgH* translocation-associated oncogenes *Bcl2*, *Myc* and *Ccnd1* could transform mouse primary B cells both *in vitro* and *in vivo*, we then utilized this system in an effort to identify other oncogenes which might cooperate with *Bcl2* and

*Myc* in transforming mouse primary B cells *in vitro*. *Bcl2*- and *Myc*-expressing primary B cells newly established using the drug selection or cell sorting methods were used for Screening 1 or Screening 2, respectively (Figure 3A,B). Cells expressing *Bcl2* and *Myc* were infected with a retroviral library expressing cDNA from SU-DHL-6, a human B-cell lymphoma cell line with the *BCL2/IgH* translocation (Figure 3C), followed by initiation of functional screening, in which the infected cells were cultured in the absence of IL-7 and ST-2 cells (Figure 3D). Retroviral library-infected

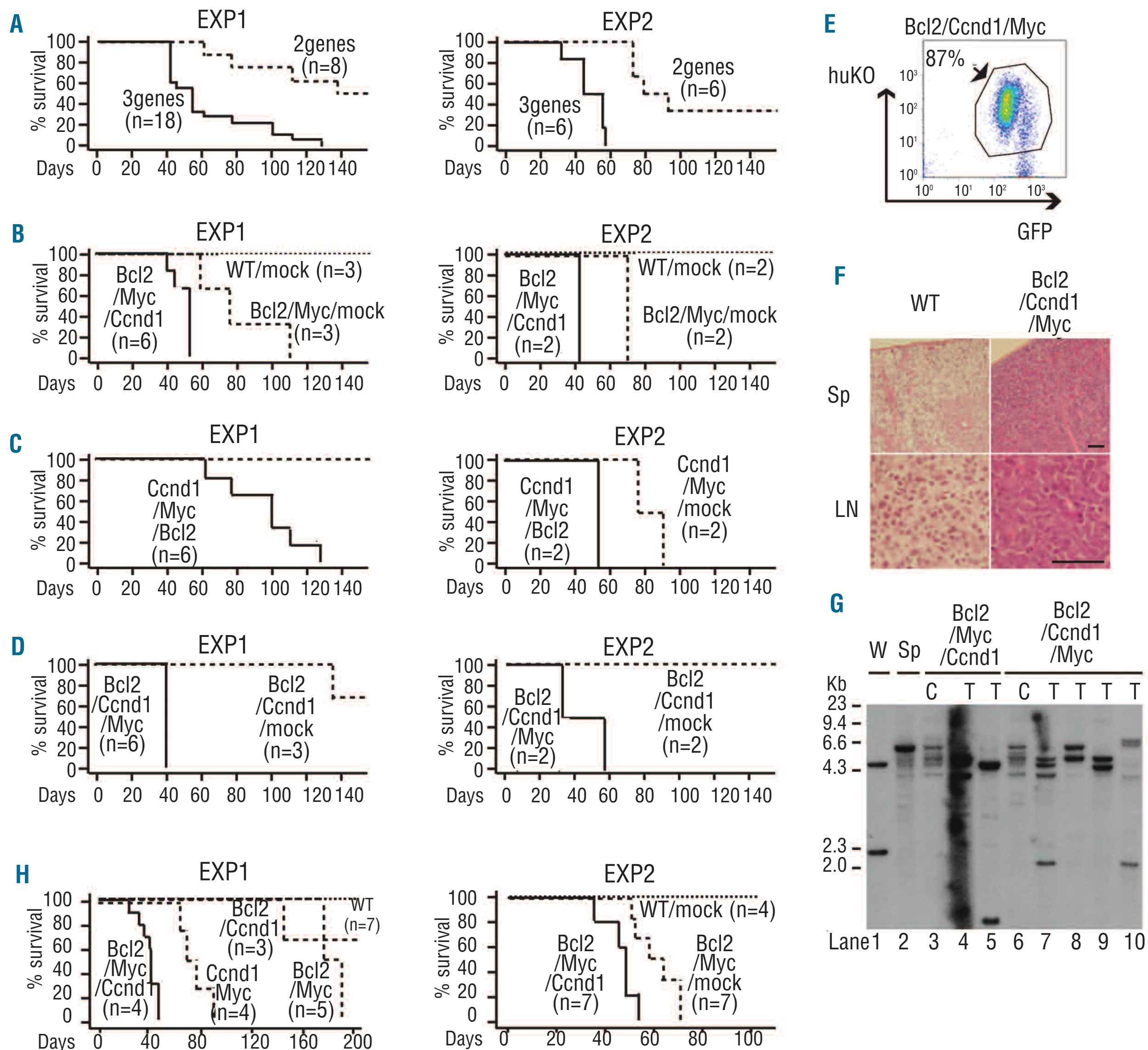


Figure 2. Enhanced tumorigenic properties of primary mouse B-cells stably expressing exogenous *Bcl2*, *Myc* and *Ccnd1*. Stable integrants established by the cell sorting method were used. (A-D) Kaplan-Meier survival curves of SCID mice transplanted with various stable integrants established by the cell sorting method. Right and left panels show the results of EXP 1 and EXP 2, respectively. “3 genes” (n=18) in EXP 1 of (A) indicates Bcl2/Myc/Ccnd1 (n=6), Ccnd1/Myc/Bcl2 (n=6) and Bcl2/Ccnd1/Myc (n=6). “2 genes” (n=8) in EXP 1 of (A) indicates Bcl2/Myc/mock (n=3), Ccnd1/Myc/mock (n=2) and Bcl2/Ccnd1/mock (n=3). “3 genes” (n=6) in EXP 2 of (A) indicates Bcl2/Myc/Ccnd1 (n=2), Ccnd1/Myc/Bcl2 (n=2) and Bcl2/Ccnd1/Myc (n=2). “2 genes” (n=6) in EXP 2 of (A) indicates Bcl2/Myc/mock (n=2), Ccnd1/Myc/mock (n=2) and Bcl2/Ccnd1/mock (n=2). In EXP 2 of (A), three-gene-expressing cells *versus* two-gene-expressing cells, *P*=0.0006. In EXP 2 of (B), Bcl2/Myc/Ccnd1 *versus* Bcl2/Myc/mock, *P*=0.0833. In EXP 2 of (C), Ccnd1/Myc/Bcl2 *versus* Ccnd1/Myc/mock, *P*=0.0833. In EXP 2 of (D), Bcl2/Ccnd1/Myc *versus* Bcl2/Ccnd1/mock, *P*=0.0896. (E) The GFP and huKO expression pattern of cells infiltrating a lymph node of a representative Bcl2/Ccnd1/Myc mouse. (F) Hematoxylin/eosin staining of spleen (Sp) and lymph node (LN) in wild-type (WT) and representative Bcl2/Ccnd1/Myc mice. Scale bar represents 50 μm. (G) Southern blot analysis of *IgH* gene rearrangements in the stable integrants established by the cell sorting method. Genomic DNA from WEHI231 (murine B-cell lymphoma cell line) (W) and spleen cells (Sp) of wild-type BALB/c mouse were used as positive controls. ”C” in lanes 3 and 6 or ”T” in lanes 4,5,7-10 denotes genomic DNA extracted from *in vitro* cultured cells or tumor cells of neoplastic lymph nodes of SCID mice transplanted with stable integrants. (H) Kaplan-Meier survival curves of SCID mice transplanted with various stable integrants established by the drug selection method. Right and left panels show the results of EXP 1 and EXP 2, respectively. In EXP 1, Bcl2/Myc/Ccnd1 cells killed SCID mice more rapidly compared to Bcl2/Myc or Ccnd1/Myc cells (*P*=0.0013, log-rank test), or Bcl2/Ccnd1 cells. In EXP 2, Bcl2/Myc/Ccnd1 cells killed SCID mice more rapidly compared to Bcl2/Myc/mock cells (*P*=0.0012, log-rank test).

cells showed robust proliferation for about 10 days (Screening 1) and for 2 to 5 weeks (Screening 2) following library infection, unlike the mock-infected cells (Figure 3E). We recovered the inserted cDNA from retrovirus-integrated genomic DNA of the proliferating cells. This process resulted in the identification of *CCND3*, *NRAS* and *RNF14* in Screening 1, and *CCND3*, *NRAS*, *PSAP* and *ASB8* in Screening 2 (Figure 3F). Of special note is that *CCND3* and *NRAS* were identified in both screenings. The recovered sequence of *NRAS* contained a missense mutation in codon 61 (Q61K), and we confirmed that SU-DHL-6 possessed the mutated *NRAS* allele.

We also conducted another experiment to confirm these results. To this purpose, we established various stable integrants using the sorting method (see *Online Supplementary Figure S1* for vector constructions and stable integrants). In the liquid culture assay without IL-7 and ST-2 cells, expression of *CCND3* or *NRAS* (Q61K) led to the stable growth of primary B cells expressing combinations of *Bcl2* and *Myc* (Figure 4A, Bcl2/Myc/CCND3 or Bcl2/Myc/NRAS), unlike the case with *RNF14*, *PSAP* or *ASB8* (*data not shown*). Furthermore, cells expressing *CCND3* and *Myc* (CCND3/Myc/mock) or *Bcl2* and *CCND3* (Bcl2/CCND3/mock) could not proliferate under the same culture conditions (Figure 4A). This led us to conclude that one of the genes (*Bcl2*, *Myc* or *CCND3*) is indispensable for the transformation of primary mouse B cells.

We then determined whether ectopic expression of *Bcl2*, *Myc* and *CCND3* or *Bcl2*, *Myc* and *NRAS* (Q61K) could transform primary B cells in a colony-forming assay. In the first plating of the methylcellulose colony assay, Bcl2/Myc/CCND3, CCND3/Myc/Bcl2 and Bcl2/CCND3/Myc cells showed more efficient colony formation than Bcl2/Myc/mock, CCND3/Myc/mock and Bcl2/CCND3/mock, respectively (Figure 4B,C). On replating, Bcl2/Myc/mock, CCND3/Myc/mock and Bcl2/CCND3/mock cells did not show any colony formation, while cells with the three genes expressing stable integrants (Bcl2/Myc/CCND3, CCND3/Myc/Bcl2 and Bcl2/CCND3/Myc) showed a higher number of colonies (Figure 4B). Moreover, Bcl2/Myc/NRAS cells formed colonies at a higher frequency than Bcl2/Myc/mock (Figure 4B). We also examined the synergistic effects of these gene combinations in the SCID transplantation model and found that Bcl2/Myc/CCND3, CCND3/Myc/Bcl2 and Bcl2/CCND3/Myc cells caused B-cell leukemia/lymphoma with shorter survival times than cells containing only two of the oncogenes [Figure 4D, cells expressing three genes (Bcl2/Myc/CCND3, n=3; CCND3/Myc/Bcl2 n=3; and Bcl2/CCND3/Myc, n=3) *versus* cells expressing two genes (Bcl2/Myc/mock, n=3; CCND3/Myc/mock, n=2; and Bcl2/CCND3/mock, n=2), $P\leq0.0001$; Figure 4E, Bcl2/Myc/CCND3 *versus* Bcl2/Myc/mock, $P=0.0246$; Figure 4F, CCND3/Myc/Bcl2 *versus* CCND3/Myc/mock, $P=0.0643$; Figure 4G, Bcl2/CCND3/Myc *versus* Bcl2/CCND3/mock, $P=0.0645$]. Bcl2/Myc/NRAS cells also killed SCID mice with an associated shorter survival time than Bcl2/Myc/mock cells ($P=0.0224$, Figure 4E). These findings confirmed that ectopic expression of *Bcl2*, *Myc* and *CCND3* or *Bcl2*, *Myc* and *NRAS* (Q61K) can function synergistically to transform primary B cells into highly aggressive malignant cells. The available expression microarray data obtained from a public database also suggested that *BCL2*, *MYC* and *CCND3* function synergistically and contribute to the histological transformation of some cases of human follicular lymphoma (*Online Supplementary Figure S5*).

### Expression microarray analysis to identify significant up- or down-regulated signaling pathways in cells expressing three genes compared to those expressing two genes

In order to investigate the deregulated pathways in the cells expressing three genes, expression microarray was employed using the stable integrants established by the cell sorting method in EXP 1 and the GSEA program with gene sets of canonical pathways. Twenty-six and 125 gene sets were significantly up- or down-regulated, respectively, in Bcl2/Myc/Ccnd1 cells (nominal $P$ value <0.05 and false discovery rate q-value <0.25) relative to Bcl2/Myc/mock cells (*Online Supplementary Table 2A,B*). The five gene sets with the most up-regulated normalized enrichment score (NES) included three muscle contraction-associated gene sets (*Online Supplementary Table S2A*, REACTOME_SMOOTH_MUSCLE_CONTRACTION, KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION and REACTOME_MUSCLE_CONTRACTION). The five gene sets with the most down-regulated NES included four translation-associated gene sets (*Online Supplementary Table S2B*; KEGG_RIBOSOME, REACTOME_PEPTIDE_CHAIN_ELONGATION, REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS and REACTOME_VIRAL_MRNA_TRANSLATION). Fifty-seven and 78 gene sets were significantly up- or down-regulated, respectively, in Ccnd1/Myc/Bcl2 cells relative to Ccnd1/Myc/mock cells (*Online Supplementary*

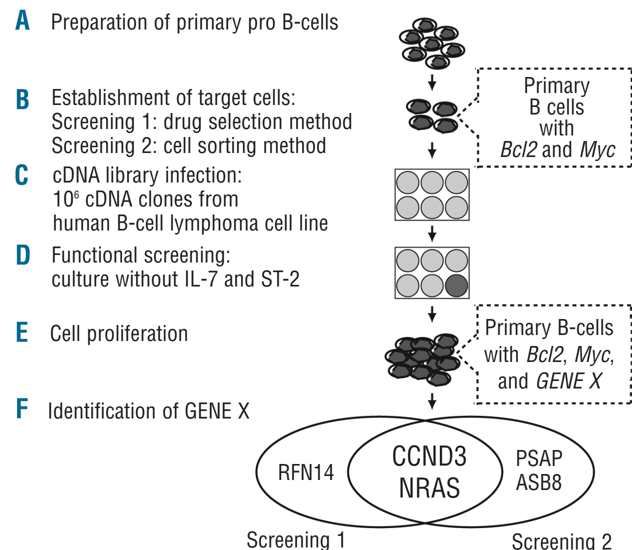


Figure 3. Diagram of the retroviral cDNA expression library screening protocol. (A) Mouse primary pro B-cells were purified from fetal liver of BALB/c mice. (B) *Bcl2* and *Myc*-expressing target cells were established by the drug selection method for Screening 1 and the cell sorting method for Screening 2. (C) Target cells were infected with a retroviral expression library containing $10^6$ cDNA from SU-DHL-6. (D) Functional screenings were performed under culture condition in the absence of IL-7 and ST-2 cells. (E) Proliferation of cells transduced with *GENE X*, which cooperates with *Bcl2* and *Myc*. (F) Inserted cDNA were recovered by PCR-amplification using the retroviral-specific primers from retrovirus-integrated genomic DNA of proliferating cells. *CCND3* and *NRAS* were identified in both screenings.

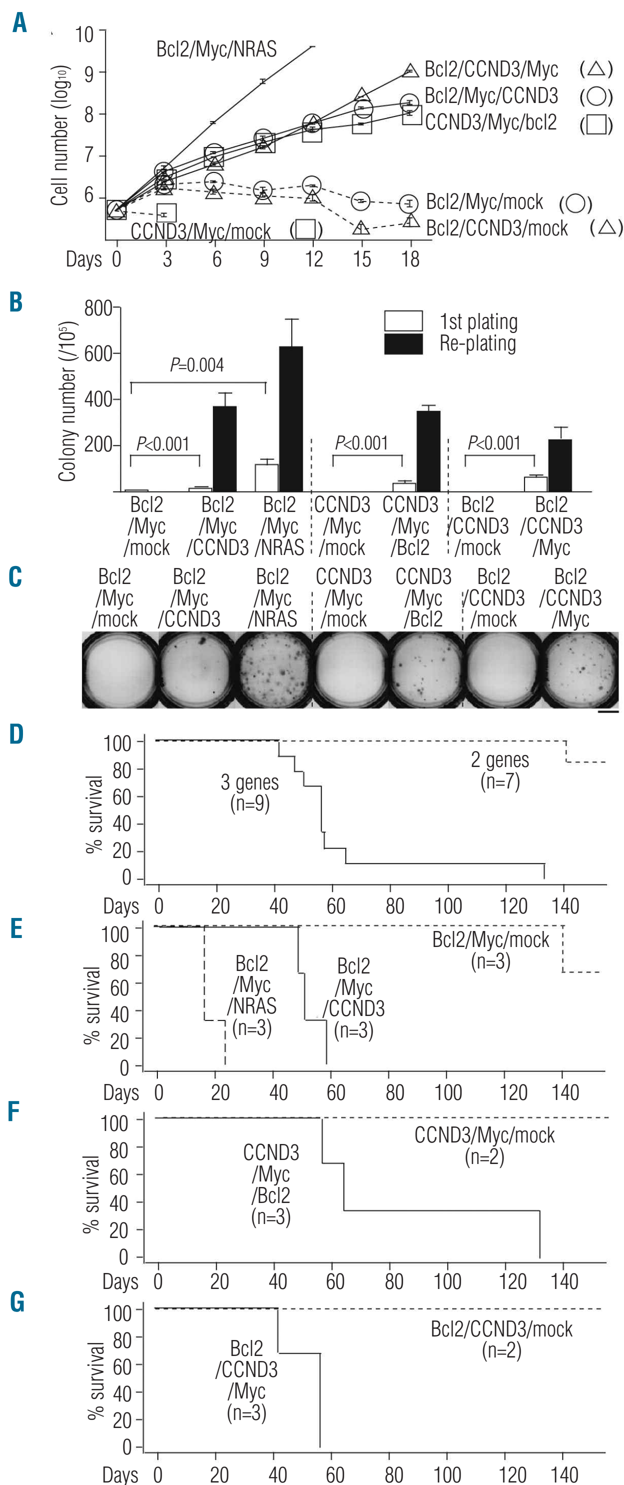


**Figure 4. Experiments confirming the synergistic effect of *CCND3* or *NRAS* with *Bcl2* and *Myc*. (A) *In vitro* growth curves of various stable integrants in the absence of IL-7 and ST-2 cells. Data are the mean ± s.e.m. (triplicate). (B) Methylcellulose colony-forming abilities of various stable integrants. Data are the mean ± s.e.m. (triplicate). *P* values are two-sided (Student's t test). (C) Photograph of representative colonies of various integrants in the first plating. Scale bar represents 10 mm. (D-G) Kaplan-Meier survival curves of SCID mice transplanted with various stable integrants. "3 genes" (n=9) in (D) indicates Bcl2/Myc/CCND3 (n=3), CCND3/Myc/Bcl2 (n=3) and Bcl2/CCND3/Myc (n=3). "2 genes" (n=7) in (D) indicates Bcl2/Myc/mock (n=3), CCND3/Myc/mock (n=2) and Bcl2/CCND3/mock (n=2).**

*Table S2C, D*). All of the five gene sets with the most up-regulated NES comprised translation-associated gene sets (*Online Supplementary Table S2C*). The five gene sets with the most down-regulated NES included three cell cycle-associated gene sets (*Online Supplementary Table S2D*; REACTOME_MITOTIC_M_M_G1_PHASES, REACTOME_CELL_CYCLE_MITOTIC and REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1). One hundred and fifty-two and 83 gene sets were significantly up- or down-regulated, respectively, in Bcl2/Ccnd1/Myc cells relative to Bcl2/Ccnd1/mock cells (*Online Supplementary Table S2E,F*). The five gene sets with the most up-regulated NES comprised translation-associated gene sets (*Online Supplementary Table S2E*). The five gene sets with the most down-regulated NES comprised cell surface receptor-associated gene sets (*Online Supplementary Table S2F*). This analysis did not show any gene sets which were commonly up- or down-regulated in all variants of the cells expressing three genes (*Online Supplementary Table S2*).

In addition to the mRNA analysis, the protein expression of some selected genes was examined. It is known that a failure to induce senescence underlies the transformation process of some mouse primary cells. We performed western blot analysis to assess expression of senescence-associated proteins, including p53, p21, p16 and p27 and cytochemical staining of senescence-associated β galactosidase. We were not, however, able to show any differences between cells expressing three or two genes (*data not shown*).

## Discussion

In this study, we demonstrated for the first time that the *IgH* translocation junction B-cell lymphoma oncogenes *Bcl2*, *Myc* and *Ccnd1* function synergistically to transform mouse primary B cells into highly aggressive malignant cells. The transformation of primary mouse B cells could be induced *in vitro* by the exogenous introduction of all three genes. Transplanted cells ectopically expressing all three genes killed recipient mice more rapidly than those expressing any combination of two of the three genes. These findings provided direct evidence that the synergic effect contributed to lethal tumor development in mice, thus increasing our understanding of human B-cell lymphoma development. Importantly, there are reports of some cases of human B-cell lymphoma possessing concurrent *BCL2*, *MYC* and *CCND1* translocations.[14,15] Our results strongly support the notion that *BCL2*, *MYC* and *CCND1* can operate synergistically in the malignant processes related to these cases.

Although cases involving a triple translocation of *BCL2*, *MYC* and *CCND1* are rare, concurrent double translocations, especially of *BCL2* and *MYC*, have been occasionally identified in cases of human B-cell lymphoma.[3-7] It is possible that lymphoma cases with deregulated expression of *BCL2* and *MYC* might have a cooperative genetic alteration which can substitute for *CCND1*. Based on such a hypothesis, we performed functional screening for genes cooperating with *Bcl2* and *Myc* using $10^6$ candidate cDNA clones of the retroviral SU-DHL-6 cDNA expression library. Through the screening, we not only identified mutated *NRAS*, one of the best-known oncogenes, but also *CCND3*.

It was previously reported that *CCND3* is recurrently, but infrequently, targeted by *IgH* translocations.[27,28] Moreover, it should be emphasized that we previously reported that *CCND3* was the putative oncogene at the 6p21 gain/amplification region frequently found in human B-cell lymphomas.[29] Although many other reports have proposed that *CCND3* is involved in human B-cell lymphomagenesis,[30-34] no direct evidence for its involvement has been provided. To the best of our knowledge, our study has provided the first direct evidence that *CCND3* can participate in the process of malignant transformation of primary B cells. Since it is well known that CCND1 and CCND3 share a redundant role,[35,36] our findings may imply that any of several genes involved in cell cycle regulation could has a synergistic effect in combination with *Bcl2* and *Myc* on human B-cell lymphomagenesis.

Our screenings picked up the *NRAS* cDNA sequence containing the missense mutation in codon 61 (Q61K). We could confirm the presence of the mutated *NRAS* allele in SU-DHL-6. Mutated *Nras* has been implicated in a minority of E-mu-myc mouse lymphomas.[37] However, mutated *NRAS* is unlikely to be the third cooperative gene acting with *BCL2* and *MYC* in human B-cell lymphoma since it is well known that human B-cell lymphomas rarely have such mutations.[38]

As shown in Figure 2G, the transplantation of polyclonal integrants expressing *Bcl2*, *Myc* and *Ccnd1* into mice resulted in tumor development with one to several clonal *IgH* rearrangements. Figure 1C shows that only a limited cell population of integrants expressing *Bcl2*, *Myc* and *Ccnd1* could form colonies, suggesting that an additional unknown gene or genes cooperating with *Bcl2*, *Myc* and *Ccnd1* may further contribute to B-cell transformation. This notion has some credence given that human cases reported to possess *BCL2*, *MYC* and *CCND1* translocations also harbored additional *BCL6* translocations.[14,15] It is important to determine whether *BCL6* and/or the other cooperative genes are necessary for human lymphoma formation.

The stromal microenvironment plays an important role in lymphoma formation *in vivo*.[39-41] Lymphoma cells escaping the influence of the microenvironment *in vivo* could mimic the synergism which we observed under the culture condition without IL-7 and ST-2 stromal cells *in vitro*, while it remains to be determined whether lymphoma cells under the influence of the microenvironment *in vivo* could mimic the synergism. In this context, it is important to note that we were able to identify some cases of histologically transformed follicular lymphoma which showed increased expression of *MYC* and *CCND3* (*Online Supplementary Figure S5*). Further studies are needed to determine whether co-activation of *BCL2*, *MYC* and *CCND3* affect the relationship between follicular lymphoma cells and their microenvironment.

GSEA was utilized in an effort to identify deregulated pathways which might be implicated in the synergistic effect of *Bcl2*, *Myc* and *Ccnd1*. Many pathways were deregulated in Bcl2/Myc/Ccnd1, Ccnd1/Myc/Bcl2 and Bcl2/Ccnd1/Myc cells relative to Bcl2/Myc/mock, Ccnd1/Myc/mock and Bcl2/Ccnd1/mock cells, respectively. Translation-associated pathways were significantly down-regulated in Bcl2/Myc/Ccnd1 cells and up-regulated in Ccnd1/Myc/Bcl2 and Bcl2/Ccnd1/Myc cells. We were unable to identify gene sets which were commonly deregulated in all variants of the cells expressing three genes. This may suggest that the synergistic effect of *Bcl2*, *Myc* and *Ccnd1* is not regulated by changes in mRNA expression but by changes in protein stability and/or modification. Further studies should provide important insights into the downstream processes associated with the synergistic effect of *Bcl2*, *Myc* and *Ccnd1*.

The studied cells expressing three genes showed some minor variations in their response to the cell growth assays without IL-7 and ST-2, and considerable differences in their response to the colony-forming and *in vivo* assays. These variant cell types were established using different retroviral vectors, and thus the cells ectopically expressed different amounts of BCL2, MYC and CCND1 proteins (*Online Supplementary Figure S3*). These differences in protein expression may have affected the results of the *in vitro* and *in vivo* assays. However, it is important to note that all cell variants expressing three genes showed a malignant phenotype in every assay, relative to the respective control cells expressing two genes.

In this study, we were able to identify cooperative oncogenes that act with *Bcl2* and *Myc* on the basis of SU-DHL-6 retroviral cDNA library screening. The use of other cDNA libraries may facilitate the identification of other cooperative oncogenes and/or microRNA. The use of siRNA or shRNA libraries could enable the identification of cooperative suppressor oncogenes. Our synergism-based screening strategy could be useful in both *in vitro* and *in vivo* studies. In our preliminary study, we recurrently identified the *TCL1A* gene as a gene that cooperates with *Bcl2* and *Myc* (*data not shown*). Our synergism-based functional analysis method is useful not only for the identification of hitherto unknown genes, but also for the identification of oncogenic synergisms with previously identified cancer-causing genes and microRNA, which may expand our systemic understanding of a wide range of cancer-causing elements. However, the method used in our study also selected pseudo-positive genes, suggesting that the method requires further improvement.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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