Published in final edited form as:

Exp Hematol. 2008 February ; 36(2): 128–139.

Bcl2 enhances induced hematopoietic differentiation of murine embryonic stem cells

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Abstract

Bcl2 is a potent antiapoptotic gene which can increase the resistance of adult bone marrow hematopoietic progenitor cells to lethal irradiation and thereby preserve their ability to differentiate. However, the effect of Bcl2 on murine embryonic stem (ES) cells induced to undergo hematopoietic differentiation in the absence of a toxic stress is not known. To test this, murine CCE-ES cells that can be induced to undergo hematopoietic differentiation in a two step process that results in upregulation of Bcl2 were used. Upregulation of Bcl2 precedes formation of hematopoietic embryoid bodies (EB) and their further differentiation into hematopoietic colony forming units (CFUs) when plated as single cells in methylcellulose. ES cells stably expressing a Bcl2 siRNA plasmid to “knock-down” endogenous expression or cells expressing WT Bcl2 or phosphomimetic Bcl2 mutants were examined. ES cells expressing the Bcl2 siRNA or those expressing a dominant-negative, non-phosphorylatable Bcl2 display a strikingly reduced capacity to form hematopoietic EBs and CFUs compared to cells expressing WT or phosphomimetic Bcl2 that demonstrate an increased capacity. Bcl2's effect on induced-hematopoietic differentiation of ES cells does not result from either decreased apoptosis or a reduced number of cells. Rather, Bcl2-enhances hematopoietic differentiation of ES cells by up-regulating p27, which results in retardation of the cell cycle at G1/G0. Thus siRNA silencing of p27 reverts Bcl2's enhancement phenotype in a manner similar to that of Bcl2 “silencing” or expression of a nonphosphorylatable Bcl2. In addition to Bcl2's well described antiapoptotic and cell cycle retardant effect on somatic cells, Bcl2 may also function to enhance induced hematopoietic cell differentiation of murine ES cells. These findings may have potential relevance for expanding hematopoietic stem/progenitor cell numbers from an ES cell source for stem cell transplantation applications.

Keywords

Embryonic Stem (ES) cells; hematopoietic differentiation; Bcl2; phosphorylation

Introduction

Bcl2 family members have been reported to regulate somatic cell differentiation but little is known about whether or how these apoptotic genes may be involved in embryonic stem cell

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Author contributions: Yan-Yi Wang – performed the research and wrote the paper Xingming Deng – created the Bcl2 mutants and analyzed the data Lijun Xu and Fengqin Gao and Tammy Flagg – collected the data W. Stratford May – oversaw entire project and wrote/edited the paper.

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(ES) differentiation. Some antiapoptotic members including Bcl2, BclxL and MCL1 [1,2] have been reported to support neuronal and sensory neuron cell differentiation and maturation [3-5], and have a role in restricting lineage choice during differentiation of multipotent hematopoietic progenitor cells [6,7]. Interestingly, inhibition of Bcl2 expression by antisense oligodeoxynucleotide technology has been reported to block neuronal differentiation but the mechanism is not clear [3]. Bcl2 has also been reported to influence the morphological transition of “undifferentiated” ES cells to “committed” precursor cells in both adult and embryonic non-hematopoietic tissues [8]. Collectively these reports suggest that Bcl2 may be involved in regulating/influencing cell differentiation as well as tissue fate through its antiapoptotic function. Indeed, expression of exogenous Bcl2 in adult hematopoietic stem/progenitor cells of transgenic mice mediates enhanced resistance to irradiation [9]. Although Bcl2 is not expressed in early adult hematopoietic stem cells [10], when exogenous Bcl2 is expressed it can function to protect these cells against stresses that induce apoptosis. However, Bcl2's effect on hematopoietic differentiation of ES cells, if any, is not known.

Recently it was discovered that phosphorylation is required for Bcl2's functional antiapoptotic and cell cycle retardant activity and its role in delaying DNA damage repair [11-15]. Dynamic phosphorylation of Bcl2 occurs on the S70 site located in the flexible loop domain (FLD). Phosphorylation can also occur on two other potential sites, T69 and S87 which are also located in the FLD. Either mono or multiple site phosphorylation of Bcl2 enhances its function [11-16]. Importantly, Bcl2 phosphorylation at S70 is physiologically induced by growth factors that mediate cell growth and survival including IL-3, erythropoietin and NGF [17-20]. Regardless of the inducing agent, Bcl2 phosphorylation enhances its antiapoptotic function since the nonphosphorylatable S70A or the AAA Bcl2 mutant displays a loss of function phenotype and fail to block apoptosis or retard cell cycle progression [11].

While not yet clinically achievable, ES cells are envisioned to represent a novel and potentially limitless source of functional hematopoietic stem and progenitor cells for transplantation of patients with bone marrow failure diseases including leukemia and aplastic anemia who may not have a living, related or unrelated donor match. Therefore, we tested the effect of silencing endogenous Bcl2 expression or expressing wild type (WT), phosphomimetic or nonphosphorylatable Bcl2 mutants on induced hematopoietic differentiation of murine CCE-ES cells. Results reveal that Bcl2 has a stimulatory effect on stem cell fate during induced-hematopoietic differentiation that is not the result of its potent antiapoptotic function.

Materials and methods

Retroviral construction, production, and transduction

Murine Bcl2 cDNA was cloned in pUC19 plasmid. Nucleotides corresponding to each serine (S) or threonine (T) residue were substituted to create a conservative alteration to alanine (A) or glutamic acid (E) with a site-directed mutagenesis kit (Clontech) as described [18]. Each single mutant was confirmed by sequencing the cDNA and subsequently cloning into the pMigR1 retroviral expression vector [21]. The MIGR1 retrovirus contains the murine stem cell virus (MSCV) promoter and an internal ribosomal entry site (IRES) element followed by the GFP gene. The MIGR1 vector expresses both the Bcl2 insert and the GFP marker protein from a single IRES-containing message driven by the murine stem cell virus retroviral promoter/enhancer (Fig. 2A).

Retroviral supernatants were generated by transient transfection of BOSC23 cells and titered on NIH 3T3 cells before use [22]. These were immediately used for transduction of murine ES cells (CCE) on gelatinized six-well plates [23]. The infected ES cells were traced by their expression of green fluorescence protein (GFP) and sorted using FACS. Stably expressing batch cultures of ES cells were expanded and used in the following experiments.

Vector-based gene silencing of Bcl2 or p27 using RNA interference

Bcl2 or p27 gene target sequence GCTGCACCTGACGCCCTTC or GTGGAATTTTCGACTTTCAG was identified as a template for producing the siRNA as determined using the Ambion siRNA Target Finder and the mouse Bcl2 or p27 cDNA sequence. The Bcl2 or p27-specific hairpin siRNA insert (sense-loop-antisense) was determined using a computerized insert design tool based on a target sequence following instructions on the Ambion website. The oligonucleotide encoding the Bcl2 or p27-specific hairpin siRNA insert was synthesized and ligated into the pSilencer™ 2.1-U6 hygro vector (Ambion, Austin, TX). The pSilencer™ 2.1-U6 hygro plasmid bearing the siRNA insert was used to transfect CCE ES cells using Lipofectamine™ 2000 according to the manufacturer's instructions. A scrambled control siRNA that is not homologous to any known gene was used as a negative control. Stable clones expressing Bcl2 or p27 or control siRNA are selected in hygromycin and the level of Bcl2 or p27 silence was determined by Western Blot.

Cell culture

Murine CCE-ES cells were grown on gelatinized dishes in DMEM (GIBCO, Cat.# 11965-084) supplemented with leukemia inhibitory factor (LIF), 15% FBS (GIBCO, Cat.# 16141-061), L-glutamine (Cellgro, Cat.# 25-005-CI), nonessential amino acid, and β -mercaptoethanol (Sigma). Unless otherwise stated, all reagents for cell culture and *in vitro* differentiation of ES cells were purchased from StemCell Technologies Inc ([STI], Vancouver, BC, Canada). All cell culture was performed in humidified incubators at 37°C with a mixture of 5% CO₂ in air.

Induced hematopoietic differentiation of murine CCE-ES cells

Murine CCE-ES cells can undergo induced hematopoietic differentiation *in vitro* in a two step process that represents a modification of the method described by Keller [24] and as detailed in the Technical Manual of Stemcell Technologies, Inc. (www.stemcell.com; Fig. 1A). Briefly, for Step 1 induced-hematopoietic differentiation, murine CCE ES cells (500 cells/ml) are cultured in IMDM medium (Stemcell, Cat.# 36150) supplemented with 1% methylcellulose (Stemcell, Cat.# 03120), 15% FBS (GIBCO, Cat.# 16141-061), 2mM L-glutamine (Cellgro, Cat.# 25-005-CI), 150 μ M monothioglycerol (MTG, Sigma), and 40 ng/ml murine SCF plated in low adherence 35mm dishes (StemCell Technologies Inc., Cat. 27100). The cultures are placed into a covered petri dish along with an open 35 mm dish containing sterile water for humidification and incubated at 5% CO₂ at 37° C. Cultures are re-fed on day 6 with “feed medium” containing 10 μ g/ml SCF, IL-3, human IL-6 and 3U/ml erythropoietin as described in the manual (see above). For Step 2, the resulting day 11 embryoid bodies (EBs) that develop are collected and made into a single cell suspension that is plated at 2×10^4 cells/ml in methylcellulose-based, hematopoietic “differentiation medium” containing SCF, IL-3, IL-6, erythropoietin and insulin and transferrin in 35 mm dishes. Cultures are incubated in 5% CO₂ in 37 ° C at 95% humidity for 10 days in order to enumerate hematopoietic colonies (CFUs) that form, including BFU-E, CFU-GM and CFU-MIX. The EBs formed during Step 1 are scored as “hematopoietic” or “non-hematopoietic” EBs. “Hematopoietic” EBs are morphologically identified using a dissecting microscope by the visual presence of macrophages, erythroid cells and occasionally granulocytic cells at the edges of the EB. Hemoglobinization of the erythroid cells is often visible. The “Efficiency of EB formation” = Total EBs scored per dish/ Number of ES cells plated per dish. The “% hematopoietic EBs” = Number of Hematopoietic EBs scored per dish/Total number of EBs identified per dish. Hematopoietic differentiation was further documented and quantified by the percentage of CD41⁺ cells that formed in the EBs during Step 1 of induced-hematopoietic differentiation. The CFUs formed during Step 2 are identified and scored visually using a microscope as defined in the manual.

Fluorescent staining and FACS analysis

Day 11 EBs from Step 1 are disaggregated into single cell suspensions by washing with PBS and resuspending in 0.1% trypsin in PBS and pipetting the mixture up and down. The cells are strained through a filter mesh (Miltenyi Biotec, Cat. # 130-041-407) to remove clumps and collected by low speed centrifugation at $300 \times g$.

For the Cell Viability Assay, Annexin V-CY5 (BD Pharmingen, San Diego CA.) and 7-AAD (Sigma) or propidium iodide (PI; Sigma) staining was done at room temperature for 15 min according to the manufacturer's specifications (BD Pharmingen, San Diego CA.). For dual staining of Annexin V-CY5 and CD41-PE (Santa Cruz Biotechnology), after Annexin V-CY5 staining, cells were transferred to 4°C, and CD41-PE antibody was added. After 20 min, samples were diluted with annexin V binding buffer containing 7-AAD and analyzed by FACS [25].

For hematopoietic differentiation and cell cycle analysis, single viable cells were separated by Ficoll centrifugation (Amersham Biosciences, Sweden) and stained with a primary SSEA-1 antibody (Chemicon, Cat.# MAB 4301) for 30 minutes at 4°C after blockade of the Fc receptor using mCD16/CD32 (BD Biosciences Pharmingen), a secondary mouse IgM-APC antibody (BD Biosciences Pharmingen) was added and incubated for 30 minutes at 4°C. Simultaneously, cells were stained with a CD41-PE antibody used to assess hematopoietic differentiation as reported [25]. Cells were fixed in 90% methanol for 60 minutes at 4°C and stained with 50 µg/ml Propidium Iodide (Sigma) to determine cell cycle distribution.

For detection of phosphorylated Bcl2, Ficoll separated cells were fixed and permeabilized using BD Cytofix/Cytoperm Kit according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, Cat.# 554715). Permeabilized cells were incubated with 0.5% BSA in PBS for 10 minutes and stained with a primary anti-Phospho-Bcl-2 (Ser70) specific antibody (Cell Signaling, Cat. # 2871) followed by incubation with a secondary PE-conjugated donkey anti-rabbit antibody for 30 minutes at 4°C. Cells were washed and analyzed by FACS. FDC-P1/ER cells expressing Bcl2 were incubated with 10 nM Bryostatin 1 (sigma) to induce Bcl2 phosphorylation [17] and used as a positive control for the phospho-specific antibody.

Western blotting

Cells were washed with ice-cold phosphate-buffered saline (PBS) and homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0; 0.5% NP-40; 1 mM EDTA; 150 mM NaCl; 10% glycerol; 1 mM Sodium Vanadate; 50 mM Sodium Fluoride; 10 mM Sodium Pyrophosphate; 1 mM β-Mercaptoethanol) containing a protease inhibitor cocktail set I (Calbiochem, Cat.# 539131). Cell extracts were clarified by centrifugation at 14,000 for 10 min and an aliquot of the supernatant removed for total protein determination using Bio-Rad Protein Assays. An aliquot corresponding to 100 µg of total protein is then separated by SDS-PAGE under reducing conditions and transferred electrophoretically to a nitrocellulose filter. Nonspecific binding of antibody is initially blocked by incubation of the filter in 5% nonfat dry milk containing 0.1% Tween 20 in PBS for 2 hr at room temperature. Immunoblotting was carried out using several antibodies against Bcl2, p27 and p21 (Santa Cruz Biotechnology) followed by addition of peroxidase-conjugated secondary anti-immunoglobulin antibodies. The blots were developed using the enhanced chemiluminescence method (ECL, Amersham Biosciences, England).

Results

Silencing Bcl2 using siRNA or stable expression of Bcl2 or phosphomimetic mutants enhances induced-hematopoietic differentiation of murine CCE-ES cells *in vitro*

Murine ES cells can be induced to undergo hematopoietic differentiation in a two step process (Fig. 1A). By definition, induced ES cells first form “hematopoietic EBs” during Step 1 after 11 days of culture and then, when dispersed as single cells in methylcellulose, secondary form colonies of the BFU-E, CFU-GM and CFU-MIX type in Step 2 as described in Methods. To test the effect of Bcl2 on hematopoietic differentiation, the expression of endogenous Bcl2 was efficiently silenced using siRNA technology. Since the capacity of the control or siRNA Bcl2 expressing ES cells to form EBs and the total number of EBs formed in Step 1 was not significantly changed, these data indicate that silencing of endogenous Bcl2 expression does not affect ES cell proliferation or EB formation under these conditions (Fig. 1B, C and D). However, the % of “hemopoietic” EBs (Step 1) and the number of CFU's formed per 500 ES cells plated is drastically reduced by >50% (Fig. 1E). This reduced capacity is apparently not due entirely to any increased apoptosis since EB cell viability is only reduced by ~ 12% (i.e. 78.9% of control vs. 66.5% from Bcl2 siRNA, Fig. 1F). Hematopoietic differentiation was determined using FACS analysis to compare undifferentiated ES cells in the EBs formed during hematopoietic differentiation. SSEA-1 was used as a stage-specific embryonic antigen to detect undifferentiated ES cells and CD41 expression was used to detect “hematopoietic” differentiation [25,27-28]. While only 15.7% of control cells expressed SSEA-1 and 26.3% expressed CD41, 42.7% of Bcl2 silenced cells continue to express SSEA-1 and only 7.7% continue to express CD41 (Fig. 1G). These data indicate that silencing of endogenous Bcl2 significantly inhibits induced-hematopoietic cell differentiation of ES cells.

Because silencing of Bcl2 inhibits induced-hematopoietic differentiation of ES cells, we tested whether Bcl2 phosphorylation affected this process. We compared expression of WT, mono- or multisite phosphomimetic [S70E, EEE (T69E/S70E/S87E)] or nonphosphorylatable [S70A or AAA (T69A/S70A/S87A)] Bcl2 mutants. WT or each of the Bcl2 mutants was expressed in ES cells using the MIGR1 retroviral expression system as described in the Methods (Fig. 2A). Retrovirally infected, batch cultures of ES cells that stably express similar levels of the Bcl2 transgene (Fig. 2 B and C) were then used to compare to their capacity to undergo induced hematopoietic differentiation. Importantly, during both Step 1 that leads to hematopoietic EB formation and Step 2 where CFUs are formed, the transgenes were continuously expressed as detected by both Western blot and the continued monitoring green fluorescence of cells comprising either EBs or CFUs (Fig. 2D; panel 1 represents EBs and panel 2 a CFU). Thus, any differential effect observed for Bcl2 is not considered to result from a silencing or loss of expression of the transgene during differentiation. Results reveal that cells expressing the mono- or multisite phosphomimetic Bcl2 (S70E or EEE) mutant displayed a marked increase (up to 2–3 fold) in formation of “hematopoietic” EBs and CFUs (including BFU-E, CFU-GM and CFU-MIX, Fig. 3 C and E). Again the efficiency of hematopoietic EB formation or total cell number per 500 ES cells initially plated is not significantly different (Fig. 3A and B). Furthermore, the loss of viability of CD41 expressing, differentiated cells remains 1.4% and is not altered (Fig. 3D), indicating that the effect of Bcl2 on induced-hematopoietic differentiation of ES cells is not likely due to its antiapoptotic function.

By contrast, ES cells expressing either of the nonphosphorylatable Bcl2 mutants S70A or AAA, display markedly reduced total numbers of hematopoietic EBs in Step 1 and CFUs in Step 2.

However, any effect of the Bcl2 mutants in enhancing or reducing hematopoietic EB or CFU formation does not appear to be due to a change in cell viability since the % of Annexin V expressing cells obtained from EBs is not significantly changed for any Bcl2 mutant expressing cells (Fig. 3D). In addition, the failure to express CD41 appears to be balanced by the

continuous expression of SSEA-1 by the AAA Bcl2 expressing cells from Step 1 (Fig. 3F). Again, more of the Bcl2 expressing cells display CD41 and less express SSEA-1 compared to vector-only cells, indicating an enhanced capacity of such cells to undergo induced hematopoietic differentiation (Fig. 3F). Furthermore, the EEE Bcl2 expressing cells demonstrate an even greater capacity to undergo differentiation with increased numbers of progeny expressing CD41 and a reduced number expressing SSEA-1 (Fig. 3F). By contrast the AAA Bcl2 expressing cells reciprocally show reduced expression of CD41 and increased expression of SSEA-1. This enhanced stimulatory effect of EEE Bcl2 over wt Bcl2 likely occurs because the phosphomimetic Bcl2 contains a stable charge at the physiologic phosphorylation site that is similar to that of a covalently attached phosphate, but is not subject to dynamic phosphorylation – dephosphorylation that occurs on wt Bcl2 [18,26].

Results reveal that EBs derived from vector-only, WT or EEE Bcl2 expressing ES cells develop 28.2%, 36.8%, and 69.6% CD41⁺ cells, respectively, compared to the AAA Bcl2 expressing cells that develop only 5.1% CD41⁺ cells (Fig. 3F). Importantly, cells comprising the EBs that develop from AAA Bcl2 expressing ES cells remain >50% SSEA-1 positive (Fig. 3F), demonstrating a failure to differentiate under these conditions rather than enhanced cell death. These data suggest that the nonphosphorylatable Bcl2 mutants act in a dominant-negative manner to retard/inhibit induced hematopoietic differentiation of ES cells *in vitro*. Since the EEE phosphomimetic and the AAA nonphosphorylatable Bcl2 forms display the most pronounced effects on stimulating or retarding hematopoietic EB and CFU formation, respectively, we chose to conduct further studies comparing only these two forms of Bcl2.

AAA-Bcl2 expressing ES cells undergo significantly less induced hematopoietic differentiation but appear only slightly less viable compared to vector-only or wt Bcl2 expressing cells in culture (Fig. 3C and D). However, these ES cells are able to produce similar total number cells (Fig. 3B). Therefore, we assessed the cell cycle status at various times (i.e. day 0 through day 11) of Step 1. Results reveal that the cell cycle is similar for all Bcl2 expressing cells between day 0 and day 6, but by day 7, and even more pronounced by day 11, there is a dramatic difference between vector-only, wt and EEE Bcl2 expressing cells in that they have begun to cycle more slowly. The slower proliferation at days 7–11 is associated with an increase in induced hematopoietic differentiation observed at this time (i.e. when hematopoietic EBs are appearing) (Fig. 3C and G). By contrast, AAA-Bcl2 expressing cells continue to proliferate at a similar rate to vector-only cells (Fig. 3E and G).

Expression and phosphorylation of endogenous Bcl2 occurs in a stage-specific manner during induced hematopoietic differentiation of ES cells

We could detect both expression and phosphorylation of endogenous Bcl2 during induced hematopoietic differentiation of ES cells. While Bcl2 is not expressed in undifferentiated ES cells at day 0, Bcl2 is readily detected by Western blotting on day 3 of induced-hematopoietic differentiation (Fig. 4A and B). Interestingly, in Step 1, day 3 is a time during differentiation that precedes morphological identification of cells comprising hematopoietic EBs that is characterized by acquired CD41 expression. To test the effect of Bcl2 phosphorylation on ES cell induced differentiation, we determined whether endogenous Bcl2 is phosphorylated by performing FACS analysis of EB-derived, single cells at day 6 of Step 1 using an anti-phospho-Ser70 Bcl2 antiserum (described in Methods). Results reveal that Bcl2 is phosphorylated during induced differentiation (Fig. 4C-middle panel). However, and most interestingly, the Bcl2 transgene that is exogenously expressed in undifferentiated ES cells apparently does not become phosphorylated, even following stimulation of cells with Bryostatin 1, a potent activator of a Bcl2 kinase (Fig. 4C-upper panel, 17, 19). As a positive control for Bryostatin 1, Bcl2 phosphorylation is rapidly in IL-3 dependent myeloid FDC P1/ER cells (Fig. 4C-lower panel). Collectively, these results indicate that the expression and phosphorylation status of

Bcl2 is temporally associated with induced hematopoietic differentiation of ES cells. Failure to phosphorylate Bcl2 in undifferentiated ES cells may be due to the absence or inactivity of a Bcl2 kinase(s) or to the expression a potent Bcl2 phosphatase activity that rapidly dephosphorylate Bcl2 [26,29]. This would explain, at least in part, why Bcl2 expression in ES cells does not in and of itself induce hematopoietic differentiation but only facilitates the process when the appropriate hematopoietic growth factors are added (i.e. step 1 and 2). Additional studies will be necessary to determine the mechanism in ES cells.

Expression of the cell cycle inhibitor p27 is required for ES cell induced hematopoietic differentiation

We and others have reported that Bcl2 can apparently function to retard cell cycle progression [13,30-37]. We also discovered that retardation of the cell cycle is closely associated with up-regulation of the potent cell cycle inhibitor, p27 [13]. To investigate the mechanism by which Bcl2 may enhance ES-cell induced hematopoietic differentiation *in vitro*, we first tested a role for p27 or p21, two critical cell cycle inhibitors. While the expression of p21 could not be detected in either undifferentiated ES cells or cells from day 4 induced EBs, p27 is readily detected in vector-only and phosphomimetic Bcl2 expressing ES cells by day 4–7 (Fig. 5A). Interestingly, p27 is also not detected in the AAA Bcl2 expressing cells even by day 11 (Fig. 5A) which is consistent with the failure of these cells to undergo G0/G1 cell cycle arrest and hematopoietic differentiation (Fig. 3C and G). These data support the potential role for p27 and cell cycle inhibition in facilitating induced hematopoietic differentiation of ES cells. In addition, these findings support the notion that the AAA-Bcl2 mutant may function in a dominant-negative manner to block or retard induced differentiation of ES cells.

Next, to test whether p27 expression is required for induced hematopoietic differentiation of ES cells, an siRNA “knock-down” strategy was employed to silence the expression of endogenous p27. Results reveal that p27 expression is efficiently silenced (by >90%) in vector-only, WT, and EEE Bcl2 expressing ES cells induced to differentiate (Fig. 5B). When p27 is silenced, these cells still form EBs with a similar efficiency and total number of cells (Fig. 5C and D). However, any increase in induced hematopoietic EBs and CFUs is inhibited (Fig. 5F and G). Furthermore, the effect of silencing p27 does not appear to result from a significant decrease in viability of cells undergoing induced differentiation (Fig. 5E). Also, by day 11 of induced hematopoietic differentiation the percentage CD41⁺-expressing cells in EBs is dramatically reduced in p27 silenced cells (i.e. to <2%) compared to the high level of CD41 expression in WT or EEE Bcl2 expressing cells (i.e. 36–61%; Fig. 5H). Moreover the silencing of p27 in ES cells accelerates their rate of cell cycling (Fig. 5I). These results indicate that the percentage of ES cells entering into S and G2/M phase is markedly increased in both WT and EEE Bcl2 expressing cells that have p27 “knocked-down” (Fig. 5I). Collectively, these findings support the conclusion that p27 expression is required to facilitate Bcl2 enhanced induced-hematopoietic differentiation of ES cells *in vitro*. Enhanced hematopoietic differentiation therefore appears to require Bcl2's cell cycle retardant function that is associated with p27 upregulation.

Discussion

Results demonstrate that Bcl2 expression is required for efficient, induced hematopoietic differentiation of murine CCE ES cells *in vitro*. Furthermore, WT and particularly the more functional mono- and multisite phosphomimetic Bcl2 mutants enhance induced hematopoietic differentiation when expressed in ES cells. Enhancement appears to be specific for Bcl2 since either silencing the expression of endogenous Bcl2 or forced expression of a phosphorylation deficient Bcl2 mutant fails to support induced hematopoietic differentiation. Since Bcl2 has an antiapoptotic function, viability of EBs and CFUs change as a consequence of Bcl2 silencing

or expression, which explains these findings. While results do indicate that “knock-down” of endogenous Bcl2 expression in induced ES cells may account for 10–12% decreased viability, this can not account for the ~80% reduction in CFUs (Fig. 1F and H, respectively). Indeed, expression of wt or the EEE Bcl2 transgene may account for the 75.9% and 88.2% in viability for wt or EEE expressing cells, respectively, as analyzed by decreased Annexin-V Staining (Fig. 3D). Further, it may explain why AAA Bcl2 expressing cells show ~7% reduction in viability as compared to vector-only control cells (Fig. 3D). This degree of gain or loss in viability does not equate to the quantitative increase or decrease in hematopoietic EBs and CFUs for EEE or AAA Bcl2 expressing cells, respectively. It could, however, largely explain the increase in wt Bcl2 expressing EBs and CFUs. In addition, the mechanism by which Bcl2 is able to induce hematopoietic differentiation of ES cells *in vitro* requires the expression of the cell cycle inhibitor, p27, which functions to retard cells in G0/G1 of the cell cycle during differentiation. Presumably, p27 mediated cell cycle retardation is required for effective hematopoietic growth factor induced hematopoietic differentiation of ES cells. This conclusion is supported by results revealing that silencing the expression of endogenous p27 during hematopoietic growth factor-induced hematopoietic differentiation will abrogate not only G1/G0 cell cycle arrest but also any enhanced hematopoietic EB and CFU formation, even in ES cells expressing the potent EEE-Bcl2 mutant (Fig. 5). Importantly, loss of expression of the undifferentiation SSEA-1 marker and expression of the hematopoietic differentiation marker CD41 is dramatically increased on differentiating ES cells that express Bcl2 or a phosphomimetic Bcl2 mutant (Fig. 3F). Conversely, SSEA-1 expression is maintained while CD41 expression fails to occur on ES cells in which endogenous Bcl2 is either silenced or in those cells expressing a non-phosphorylatable Bcl2 transgene that show inhibition of induced differentiation. Collectively, these findings indicate that phosphorylation of Bcl2 is also necessary for induced hematopoietic differentiation of ES cells.

P27 has been well studied as an inhibitor of the G1→S transition in the cell cycle and its expression has been reported to facilitate embryonal carcinoma (EC) cell [30,35,37], osteoblast [31], oligodendrocyte precursor [32], and keratinocyte differentiation [36]. While the mechanism by which p27 is involved in induced hematopoietic differentiation of ES cells observed in these studies is not clear, we previously reported that Bcl2 can retard cell cycle progression in adult derived myeloid cells in a mechanism requiring p27 up-regulation and decreased intracellular ROS generation [13]. Therefore, we now propose that p27 is a necessary and specific molecular “link” in Bcl2’s ability to facilitate enhanced induced-hematopoietic differentiation of ES cells *in vitro*. Interestingly, Bcl2 has been reported to regulate both neural and adult hematopoietic progenitor cell differentiation [3-8]. Therefore, it may not be surprising that Bcl2 can facilitate/enhance hematopoietic differentiation of murine ES cells. However, since endogenous Bcl2 is not expressed in undifferentiated ES cells but is up-regulated by day 3, a time that precedes morphological or phenotype detection of hematopoietic differentiation, these data indicate that Bcl2 may play a specific role in “facilitating/enhancing” hematopoietic differentiation of ES cells. Since Bcl2 is not expressed in undifferentiated ES cells, this indicates that self-renewal of ES cells does not require Bcl2 but that expression of functional, phosphorylatable Bcl2 (i.e. wt or phosphomimetic mutation) is necessary for efficient induced hematopoietic differentiation. This conclusion is strongly supported by data indicating that while endogenous Bcl2 is first expressed by day 3 of ES cell induced differentiation, “knock-down” of Bcl2 expression completely abrogates any induced hematopoietic EB and CFUs formation (Fig. 1B, E and H). Furthermore, since expression of the non-phosphorylatable AAA/Bcl2 mutant fails to enhance and even inhibits hematopoietic differentiation (Fig. 3A-E), these data also support the notion that the nonphosphorylatable AAA Bcl2 mutant dominantly inhibits the effect of endogenous Bcl2. However, the mechanism for this dominant-negative inhibitory effect is not yet understood since the same non-phosphorylatable Bcl2 mutant does not exert a dominant-negative effect on Bcl2’s antiapoptotic or cell cycle retardant function, at least when expressed in adult myeloid cells [11].

While further studies will be required to sort out the differential effect of nonphosphorylatable Bcl2, it may simply reflect the cell context and thus be a cell type restricted effect. Also, since some CFU formation does occur when endogenous Bcl2 expression is silenced during induced hematopoietic-differentiation of ES cells, we must conclude that while Bcl2 expression may not be required, it is sufficient to facilitate and/ or enhance induced hematopoietic differentiation.

In summary, our findings support the notion of a novel function for Bcl2 in facilitating/ enhancing induced hematopoietic differentiation of murine ES cells *in vitro*. The mechanism requires expression of both Bcl2 and p27 and the retardation of the cell cycle to induce maximal hematopoietic differentiation. Based on these findings, we speculate that it may even be feasible to conditionally express a potent phosphomimetic Bcl2 in undifferentiated ES cells in order to maximally enhance their potential to undergo induced hematopoietic differentiation *in vitro*. This strategy is envisioned to potentially help increase the number of hematopoietic stem/ progenitor cells available for clinical transplantation uses.

Acknowledgments

The authors wish to acknowledge Janice Taylor for help with the manuscript preparation.

Grant Support: This work was supported by a grant from the NCI (CA 44649).

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Figure 1. ES Cell Induced Hematopoietic Differentiation Schema and the effect of Bcl2 “knock-down” using siRNA on induced hematopoietic differentiation. (A) Two step ES cell induced hematopoietic differentiation schema as described in Methods. (B) The expression of endogenous Bcl2 in CCE ES cells transfected with Bcl2 siRNA or a negative control siRNA construct as described in Methods. Bcl2 is analyzed by Western blot. (C) Efficiency of EB formation in Step 1. Values are mean $\pm$ SD for three replicates. (D) Total viable cells produced per culture (on 500 ES cells initially plated). Values are mean $\pm$ SD for three independent experiments. (E) Percentage of hematopoietic EBs formed in Step 1. Values are mean $\pm$ SD for three independent experiments. (F) Viability of cells contained in day 11 EBs as indicated

by negative Annexin V staining. (G) Flow cytometry analysis of surface expression of SSEA-1 and CD41 on cells contained in day 11 EBs. The percentage of viable and Annexin apoptotic cells expressing SSEA-1 and CD41 is represented. (H) Number of CFUs formed in Step 2 normalized for 500 ES cells initially plated. Values are mean $\pm$ SD for three independent experiments.

Figure 2.

Stable Bcl2 transgene expression in murine CCE ES cells during hematopoietic differentiation. (A) Map of retroviral Expression Vector MigR1 containing Bcl2 transgene. (B) Stable expression of Bcl2 transgenes in ES cells determined by western blotting. (C) Western blot analysis of Bcl2 transgene expression in day 0 ES through day 11 EB cells. (D) Stable expression of GFP in cells comprising day 11 EBs (1) and in the day 10 hematopoietic CFUs (2) derived in Step 2. Expression of GFP in cells of EBs and CFUs indicates stable expression of the Bcl2 transgenes. Note: The colonies formed by cells expressing AAA Bcl2 in differentiation Step 2 are EB(1) but few can be induced to hematopoietic CFUs in Step 2 (2).

Figure 3.

Effect of stable expression of Bcl2 mutants on induced hematopoietic differentiation of ES cells. (A) The efficiency of EB formation in differentiation Step 1. Values are mean $\pm$ SD for three replicates. (B) The total live cell number per culture (from 500 ES cells). Values are mean $\pm$ SD for three independent experiments. (C) The percentage of hematopoietic EBs identified on day 11 of differentiation Step 1. Values are mean $\pm$ SD for three independent experiments. (D) The viability of day 11 EB cells under normal culture condition is indicated by the negative Annexin V fraction. The relationship between apoptosis (Annexin V) and hematopoietic differentiation (CD41) is shown. (E) Number of CFUs formed by day 10 of differentiation Step 2 and normalized for 500 ES cells initially plated. Values are means $\pm$ SD for three independent

experiments. (F) Flow cytometry analysis of surface expression of SSEA-1 and CD41 on cells from day 11 EBs. The percentage of total cells analyzed in each quadrant is represented. (G) Cell cycle distribution at various times during Step 1 of induced hematopoietic differentiation of ES cells. The percentage of total cells analyzed in G0/G1, S and G2/M phases of the cycle are represented.

Figure 4.

Expression and phosphorylation status of endogenous Bcl2 during induced hematopoietic differentiation of CCE ES cells. (A) Expression of endogenous Bcl2 in parental CCE ES cells during differentiation Step 1 as determined by Western blot. (B) Downregulation of the embryonic marker SSEA-1 and upregulation of the hematopoietic cell differentiation marker CD41 in parental CCE ES cells at various times during Step 1 of induced hematopoietic differentiation of ES cells. Representative of three independent experiments conducted. (C) Detection of Bcl2 phosphorylation. FDC-P1/ER myeloid cells were used as a positive control and compared to results with undifferentiated ES cells expressing the WT Bcl2 transgene. Cells are treated with 10 nM Bryostatin 1 (Bryo) for 30 minutes at 37°C (upper and lower panels).

Comparison is also made to differentiating ES cells isolated on day 6 from ES cells expressing the WT Bcl2 transgene during Step1 of induced differentiation (middle panel). Cells are fixed, permeated, and stained with the anti-phospho serine 70 -Bcl2 antibody as described in Methods.

Figure 5.

Effect of Bcl2 mutant expression on p27 and p21 expression levels and effect of p27 “knock-down” using siRNA on induced hematopoietic differentiation. (A) p27 and p21 expression at various time point during Step 1 of induced differentiation of CCE ES cells. (B) The expression of p27 or Bcl2 transgenes in CCE ES cells transfected with p27 siRNA or a negative control siRNA construct as described in Methods. p27 or Bcl2 is detected by Western blot. (C) The efficiency of EB formation in differentiation Step 1. Values are mean $\pm$ SD for three replicates. (D) The total live cell number per culture (from 500 ES cells). Values are mean $\pm$ SD for three replicates. (E) The viabilities of day 11 EB cells under normal culture condition, indicated by the negative Annexin V fraction. (F) The percentage of induced hematopoietic EBs on day 11

of differentiation step 1. Values are mean $\pm$ SD for three independent experiments. (G) Total number of CFUs formed during differentiation Step 2 per 500 ES cells initially plated. Values are mean $\pm$ SD for three independent experiments as calculated in Methods. (H) Flow cytometry analysis of surface expression of SSEA-1 or the hematopoietic differentiation marker CD41 on cells from day 11 EBs during Step 1. (I) Cell cycle distribution of cells contained in day 11 EBs of differentiation Step 1.