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A senescence rescue screen identifies BCL6 as an inhibitor of anti-proliferative p19ARF–p53 signaling

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Senescence limits the proliferative capacity of primary cells in culture. We describe here a genetic screen to identify genes that allow bypass of this checkpoint. Using retroviral cDNA expression libraries, we identify BCL6 as a potent inhibitor of senescence. BCL6 is frequently activated in non-Hodgkin’s lymphoma, but its mechanism of action has remained unclear. BCL6 efficiently immortalizes primary mouse embryonic fibroblasts and cooperates with RAS in oncogenic transformation. BCL6 overrides the senescence response downstream of p53 through a process that requires induction of cyclin D1 expression, as cyclin D1 knockout fibroblasts are specifically resistant to BCL6 immortalization. We show that BCL6 expression also dramatically extends the replicative lifespan of primary human B cells in culture and induces cyclin D1 expression, indicating that BCL6 has a similar activity in lymphoid cells. Our results suggest that BCL6 contributes to oncogenesis by rendering cells unresponsive to antiproliferative signals from the p19ARF–p53 pathway.

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The proliferative capacity of most primary cells in culture is limited by the induction of senescence. This state of irreversible growth-arrest is characterized by expression of a number of senescence-associated markers, such as senescence-associated β-galactosidase, plasminogen-activator inhibitor 1 [PAI-1], p19ARF, p53, p21cip1, and p16INK4A [Sherr and DePinho 2000]. In most rodent cells, induction of the tumor suppressor genes p19ARF and p53 is critical to the induction of senescence, as mutation of either gene allows escape from replicative senescence and causes immortalization [Harvey et al. 1993; Kamijo et al. 1997]. Consistent with a critical role for the p19ARF–p53 pathway in the induction of senescence, it was shown recently that the transcriptional repressors BMI-1 and TRX-2 inhibit senescence through down-regulation of p19ARF expression [Jacobs et al. 1999, 2000]. In contrast, genetic ablation of p16INK4A alone or p21cip1 in mouse embryonic fibroblasts [MEFs] does not render cells resistant to the induction of senescence [Pantoja and Serrano 1999; Krimpenfort et al. 2001], even though enforced expression of p16INK4A or p21cip1 [McConnell et al. 1998; Dai and Enders 2000] induces a senescent-like state in many cell types.

These data suggest that the p19ARF–p53 pathway is a critical regulator of the senescence response in murine fibroblasts, whereas the p16INK4A–pRb pathway is not. Genetic inactivation of the p16INK4A or Rb tumor suppressor genes alone is not sufficient to immortalize MEFs [Krimpenfort et al. 2001; Peepér et al. 2001]. However, simultaneous ablation of both Rb and the related p107, or inactivation of all three Rb gene family members (Rb, p107, and p130) does not rescue MEFs from senescence, even though such cells have high levels of p19ARF and p53 [Dannenberg et al. 2000; Peepér et al. 2001]. These data indicate that the Rb family proteins not only act upstream of the p19ARF–p53 pathway, through regulation of p19ARF by E2F [Bates et al. 1998], but also downstream, by rendering cells insensitive to p53 signaling.

BCL6 encodes a transcriptional repressor, which is frequently activated by chromosomal translocation in non-Hodgkin’s lymphoma [Ye et al. 1993; Chang et al. 1996; Staudt 2000]. The chromosomal translocations involving BCL6 invariably affect the promoter only and leave the ORF of BCL6 intact. BCL6 is required for normal B and T-cell development [Ye et al. 1997], but its broad expression suggests that it also has a role outside of the lymphoid compartment [Bajalica-Lagercrantz et al. 1998]. Several studies have attempted to elucidate the molecular pathways that are targeted by BCL6. DNA microarray analyses have identified several targets of BCL6, including cyclin D2, but the relevance of these targets for the activity of BCL6 has not been elucidated [Shaffer et al. 2000]. Therefore, the molecular pathways that are regulated by BCL6 during oncogenesis are still unclear.

Here we use an unbiased functional approach to identify genes that override the senescence response. Unexpectedly, we find that BCL6 efficiently inhibits senescence by conferring resistance to antiproliferative signals from the p19ARF–p53 pathway.

Results and Discussion

To identify genes that allow bypass of replicative senescence, we generated conditionally immortalized MEFs using a temperature-sensitive mutant of SV40 large T antigen [Lee et al. 1995]. The tsT antigen-expressing MEFs [tsT-MEFs] are immortalized at the permissive temperature (32°C), but enter synchronously into a senescence-like state after shift to the nonpermissive temperature (39.5°C), due to rapid disappearance of T antigen (Fig. 1A,B). That these cells undergo a growth arrest reminiscent of replicative senescence is supported by the fact that several senescence markers, including p21cip1, PAI-1 and senescence-associated β-galactosidase, are induced upon shift to the nonpermissive temperature (Figs. 1B and 3A, below; data not shown). Because T antigen

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analysis. Four independent colonies were found to carry a full-length BCL6 cDNA. The BCL6 gene is frequently activated by promoter translocation in non-Hodgkin’s lymphoma and encodes a sequence-specific transcriptional repressor (Ye et al. 1993; Chang et al. 1996).

Figure 1C shows that BCL6 expressed in tsT-MEFs does not confer a growth advantage at 32°C, but selectively allows continued proliferation at 39.5°C (Fig. 1C). Importantly, when expressed in primary MEFs of FVB genetic background (at 37°C), BCL6 was very efficient in inhibiting both spontaneous senescence and premature senescence induced by a RASV12 oncogene (Serrano et al. 1997; Fig. 1D). Coexpression of BCL6 and RASV12 caused complete oncogenic transformation, as these cells also proliferated in soft agar and formed tumors in nude mice (data not shown). We have cultured both BCL6-immortalized MEFs and BCL6 + RAS-transformed MEFs for several months, indicating that BCL6 does not act merely to postpone senescence.

Induction of p19ARF expression, leading to activation of p53, is a critical step in the senescence response (Kamijo et al. 1997). To study how BCL6 inhibits senescence, we monitored the expression of the key components of the senescence pathway in BCL6-immortalized MEFs in comparison with late passage (pre-senescent) primary MEFs. Figure 2 shows that late passage primary MEFs express p19ARF protein, which causes a partial activation of p53, as evidenced by the high-level expression of the p53 target p21cip1 in these cells (Fig. 2, cf. lanes 1 and 5). Significantly, all six BCL6-immortalized MEF cell lines expressed as high levels of p19ARF as the late passage primary MEFs, indicating that BCL6 does not inhibit the induction of p19ARF expression to induce immortalization. Furthermore, BCL6-immortalized MEFs also expressed high levels of the p53 target gene p21cip1, indicating that BCL6 also does not prevent the activation of p53 by p19ARF. Together, these data suggest that activation of the p19ARF–p53 pathway, which occurs normally during the senescence response, is not suppressed by BCL6.

Establishment of MEFs as continuously growing cell lines is almost invariably accompanied by loss of the p53 or p19ARF tumor suppressors. Several lines of evidence indicate that neither p19ARF nor p53 is mutated in late passage BCL6-immortalized MEFs. First, as p19ARF mutation in MEFs invariably leads to loss of protein expression (Kamijo et al. 1997; Zindy et al. 1998), the presence
of clearly detectable levels of p19ARF in BCL6-immortalized MEFs argues that the p19ARF locus is still intact in these cells [Fig. 2]. Second, the finding that expression of p19ARF leads to expression of the p53 target gene p21\(^{cip1}\) in BCL6-immortalized MEFs indicates that both p19ARF and p53 are functional. Third, when late passage BCL6-immortalized MEFs were exposed to the DNA-damaging agent cisplatin, all six lines of immortalized MEFs were labeled with [\(^{35}\)S]methionine and lysates were precipitated with either p19ARF or p53 protein levels in BCL6-MEFs experience a dramatic reduction in p53 protein level upon shift to 39.5°C, resulting from the liberation of p53 from a T antigen complex. This, in turn, causes p53 to become active and activate its target p21cip1 or CDK4 antibody and separated on SDS–polyacrylamide gels. (Lanes 1,3) Control; (lanes 2,4) BCL6-expressing MEFs.

To examine whether BCL6 induces a sequestering protein that interacts with p21cip1, we labeled both BCL6 MEFs and control MEFs with [\(^{35}\)S]methionine and performed a low-stringency immunoprecipitation (to preserve protein interactions) with p21cip1 antibody. Figure 3B shows that a protein of ∼35 kD (indicated by bold arrow) is associated with p21cip1 in BCL6 MEFs, but to a much lesser extent in control MEFs. A protein of the same electrophoretic mobility is also more abundant in a CDK4 immunoprecipitate of BCL6 MEFs (Fig. 3B), raising the possibility that this p21cip1-associated protein is a D-type cyclin. To address this, we carried out a sequential immunoprecipitation experiment. Figure 3B shows that the 35-kD p21cip1-associated protein reacted with an antibody against D-type cyclins. Subsequent analyses indicated that the 35-kD protein is most likely cyclin D1, as the cyclin D1 protein level is up-regulated by BCL6 in three different cell types (Fig. 3C), whereas in agreement with an earlier study, cyclin D2 was down-regulated by BCL6 (Shaffer et al. 2000). This up-regulation of cyclin D1 is at the level of transcription, as cyclin D1 mRNA was also increased by BCL6 in MEFs (Fig. 3C).

To address the relevance of the observed up-regulation of cyclin D1 by BCL6, we asked whether BCL6 could immortalize MEFs derived from cyclin D1 knockout mice (Sicinski et al. 1995). Figure 4 shows that BCL6-immortalized primary MEFs from wild-type mice, but not from littermate cyclin D1\(^{−/−}\) mice. In contrast, T antigen immortalized both with equal efficiency. Importantly, coexpression of cyclin D1 allowed BCL6 to immortalize cyclin D1\(^{−/−}\) MEFs. Together, these data provide genetic evidence that cyclin D1 is a critical downstream target of BCL6 in immortalization.

To ask whether BCL6 can also extend the proliferative capacity of primary B cells in culture, we infected EBV-negative human primary tonsilar B cells with a retroviral vector expressing both BCL6 and GFP or with control GFP vector. Infected cells were subsequently cultured in the presence of CD40 ligand, IL-2, and IL-4, and the percentage of GFP-positive cells was monitored over time. Figure 5 shows that both BCL6 and control virus infected primary human B cells at an efficiency of ~2%, as judged by the percentage GFP-positive cells. In contrast to control GFP virus-infected cells, in two independent experiments, the fraction of BCL6-expressing cells increased steadily over time, indicating that BCL6-expressing B cells had a selective growth advantage [Fig. 5]. Under the culture conditions
BCL6 is unable to immortalize cyclin D1 knockout MEFs. Colony formation assay on primary MEFs at passage 5 infected with the BCL6 or BCL6 and cyclin D1 encoding retroviruses, and stained 10 d after infection. SV40 T antigen virus was used as a positive control [Large T]. (Top four dishes) cyclin D1 knockout MEFs; [bottom four dishes] genotype-matched wild-type MEFs. Growth curves of cyclin D1−/− and matched wild-type control MEFs infected with the indicated retroviruses is shown at bottom. Passage numbers indicated reflect the number of passages after retroviral infection at P = 5.

Used, primary human tonsillar B cells typically proliferate for up to 40 d. In contrast, we have been able to culture BCL6-infected B cells for over 4 mo without any signs of reduced proliferation [data not shown]. These cells were CD19+, CD5-, CD56−, indicating that they were of B-cell origin. Furthermore, they expressed both immunoglobulin κ and λ light chains, indicative of the polyclonal nature of the B-cell population [data not shown]. This indicates that expression of BCL6 also significantly extends the replicative lifespan of primary human B cells in culture. Importantly, BCL6-expressing B cells expressed higher levels of cyclin D1 protein as their mock-infected counterparts, suggesting that cyclin D1 up-regulation may also contribute to the increased proliferative capacity of BCL6-expressing human B cells [Fig. 5].

We show here that BCL6 acts as an immortalizing oncogene by rendering fibroblasts resistant to the anti-proliferative signals emanating from the p19ARF–p53 pathway during the senescence response. We find that BCL6 circumvents these antiproliferative signals, at least in part, through up-regulation of cyclin D1 expression. This is critical for BCL6-immortalizing activity, as cyclin D1 knockout MEFs specifically resist BCL6-mediated immortalization. Our work adds BCL6 to a short list of oncogenes that strictly require cyclin D1 as a downstream target to mediate their oncogenic effects. Other genes that depend on cyclin D1 for oncogenic activity include ras and neu, whereas oncogenes like c-myc and Wnt-1 do not require cyclin D1 as a downstream target [Robles et al. 1998; Yu et al. 2001]. However, our data by no means rule out that BCL6 has targets other than cyclin D1 in suppression of senescence.

The defect in germinal center formation of BCL6 knockout mice and the finding that BCL6 regulates genes involved in lymphocyte physiology have suggested that BCL6 regulates lymphocyte differentiation and the immune response [Ye et al. 1997; Shaffer et al. 2000]. Our study suggests that during lymphomagenesis, BCL6 does not act primarily to regulate differentiation, but targets the p19ARF–p53 pathway. The importance of the p19ARF–p53 pathway in lymphomagenesis is illustrated by the finding that both p19ARF and p53 knockout mice have a high incidence of spontaneous lymphomas [Donehower et al. 1992; Kamijo et al. 1997]. Suppression of this pathway may therefore contribute significantly to lymphomagenesis. Consistent with this, p19ARF−/− deficient murine preB cells proliferate indefinitely in vitro [Randle et al. 2001]. Furthermore, several studies have found an absence of p53 mutations in BCL6-expressing human lymphomas [Ariatti et al. 2000]. This suggests that these lymphomas have a reduced requirement to acquire mutations in p53, which is consistent with our finding that BCL6 acts downstream of p53. It is also important to point out that expression of BCL6 is not restricted to the lymphoid compartment only, which points at a broader role for BCL6 [Bajalica-Lagercrantz et al. 1998]. We have observed expression of BCL6 in breast cancer [van’t Veer et al. 2002] and SAGE databases provide further evidence that BCL6 is expressed in tumors of nonlymphoid origin. Thus, BCL6 may also act as an oncogene in solid tumors.

The p19ARF–p53 pathway is activated in a variety of cell types in response to a number of cellular stresses, including senescence. It has been suggested that the senescence response results primarily from the stress that cells experience when cultured in vitro [referred to as culture shock; Sherr and DePinho 2000]. The isolation of a bona fide human oncogene in our genetic screen validates the cell system that we have developed and sug-
gests that the stress that lymphoid cells experience during lymphomagenesis may be very similar to that strongly involved in vitro by the senescence response.

Materials and methods

Generation of MEFS expressing temperature-sensitive large T antigen

Primary Balb/c MEFs were infected at passage 2 at 32°C with pMEVTS retrovirus (Lee et al. 1995). G418-resistant colonies were assayed for the expression of large T antigen by Western blot analysis. Positive colonies yielding the lowest number of spontaneous background colonies at the nonpermissive temperature (39.5°C) were chosen for the functional genetic screens.

Retroviral rescue screens

To identify genes that allow bypass of senescence at the nonpermissive temperature, we infected cT-MEFs at 32°C with retroviral cDNA libraries (Barak et al. 2000). One day post-infection, the cells were split 1 in 12, allowed to attach at 32°C, and then transferred to 39.5°C. Several individual colonies were visible as soon as 8 h after temperature shift. The colonies were picked and expanded. Recovery of integrated proviruses for second-round selection has been described (Jacobs et al. 2000). After second-round selection, retrovirally encoded cDNAs were recovered by PCR with retrovirus-specific primers and identified by sequence analysis.

Cell culture, growth curves, and retroviral infection

All cells were maintained in DMEM supplemented with 10% FCS. For immortalization assays and growth curves, primary FVB MEFs were infected with either empty pBabe-Hygro vector, or vectors encoding BCL6 or H-RASG12D. Four days after infection, 2.5 × 10^5 infected cells were plated into 12-well plates in duplicate. At various time points, cells were processed for colormetric analysis of cell proliferation as described (Serrano et al. 1997). Values were normalized to the optical density at day 0. Phoenix packaging cells were used to generate ecotropic retroviruses as described (Serrano et al. 1997). Low passage (p1–p3) MEFs were used to infect with viral supernatant supplemented with 4 mg/mL polybrene. Cyclin D1 knockout MEFS (Scicsnis et al. 1995) and matched wild-type littermate controls of C57/BL6 genetic background were used at passage 5 for immortalization experiments.

Western blotting, kinase assays, and immunoprecipitations

Cell extracts were prepared in RIPA lysis buffer, assayed for protein concentration, and 20 µg of extract was resolved on 10%–12% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes. Primary antibodies used for Western blotting were C-19 for p21WAF1/CIP1, H-295 for cyclin D1, M-20 for cyclin D2, M-20 for cyclin E, and H-135 for PAI-1, all from Santa Cruz and M7211 (Dako) for BCL6. The p122 and p419 antibodies were used for detection of p53 and T antigen, respectively. Enhanced chemiluminescence (Amersham) was used for detection of proteins.

Radioactive labeling of cells and immunoprecipitation has been described previously (Shvarts et al. 1996). After labeling, lysates (in NETN buffer) were used for immunoprecipitation with either C-19 p21WAF1/CIP1 antibody or a mix of H-22 and C-22 CDK4 antibodies (Santa Cruz). For sequential immunoprecipitations, antibody complexes were disassociated by boiling in 1% SDS for 5 min. Released proteins were diluted in RIPA lysis buffer and precipitated with C-19 for p51, P-10 for PCNA, M-20 for D-type cyclins, or a mix of H-22 and C-22 for CDK4.

Immune-complex kinase assays were performed as described (Dulic et al. 1992) by use of cyclin E M-20 antibody (Santa Cruz) and histone H1 as a substrate.

For Northern blotting, total RNA was extracted with RNAzol (Tel Test), separated on 1% agarose gel, transferred to nitrocellulose, and hybridized with murine cyclin D1 cDNA probe.

Primary human B cell isolation and culture

Human tonsils were obtained from tonsillectomies. T cells were depleted by use of anti-CD4 and anti-CD8 microbeads. Next, cells were incubated with anti-CD19 FITC conjugated and anti-CD3 phycoerythrin (PE) conjugated, followed by sorting of the CD19^+ CD3^- population. The resulting B cells were 95%–98% pure upon reanalysis. B cells were cultured in Iscove's medium together with 10% FCS at 37°C as described (Banchereau et al. 1991). Briefly, CD40L-expressing L cells, 80 Gray irradiated, were seeded 5.10^5 cells per well in 24-well plates. The 5.10^5 sorted B cells were added together with IL-2 (20 U/mL) and IL-4 (50 ng/mL). After 1 wk, the cells were used for retroviral transduction. After transduction, B cells were cultured again with irradiated CD40L-expressing L cells, IL2 and IL4.

Retroviral transduction of human B cells

Retroviral transductions of B cells were performed as described (Hemskerk et al. 1999). Briefly, 5.10^7 B cells in 0.25 mL Iscove’s medium plus 10% FCS were mixed with 0.25 mL of thawed retroviral supernatant (pLZRS-BCL6-IRE5-GFP) and were plated in fibronectin-coated non-tissue culture-treated 24-well plates, and incubated for 6 h at 37°C. Next, 0.25 mL of supernatant was removed, and 0.25 mL of fresh retroviral supernatant was added and incubated at 37°C overnight.

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