BCL6 Represses Smad Signaling in Transforming Growth Factor-β Resistance

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Abstract

Transforming growth factor-β (TGF-β) controls a wide spectrum of cellular processes. Deregulation of TGF-β signaling contributes to the pathogenesis of many diseases including cancer and autoimmune diseases. TGF-β signaling is generally mediated through intracellular signal transducers and transcription factors called Smads. Herein, we have identified the oncoprotein BCL6 as a transcriptional corepressor of tumor suppressor Smad4. BCL6 physically interacts with Smad3 and Smad4, disrupts the Smad-p300 interaction, and represses the transcriptional activity of Smad4. In accordance, B-cell lymphoma cells with a high expression level of BCL6 were found to be refractory to TGF-β antiproliferative response, whereas knockdown of BCL6 expression in B-cell lymphoma cells partially restores the TGF-β responses. This study provides strong evidence that overexpression of BCL6 contributes to TGF-β resistance in B-cell lymphoma. [Cancer Res 2008;68(3):783–9]

Introduction

Smad proteins are key intracellular signal transducers in the transforming growth factor-β (TGF-β) signaling pathways (1). Upon TGF-β stimulation, TGF-β type II receptor phosphorylates the type I receptor, which in turn, activates downstream Smad2 and Smad3. Activated Smad2 and Smad3 then form a heteromeric complex with Smad4. The Smad complex is transported into the nucleus, where it binds to DNA and/or interacts with other transcription factors as well as recruiting coactivators or corepressors on the target gene promoters (2, 3). Context-dependent recruitment of transcriptional cofactors to the Smad complex largely determines the genomic responses and physiologic outcomes of cells in response to TGF-β.

BCL6, involved at breakpoints of chromosomal translocation affecting band 3q27, encodes a transcriptional repressor (4). Chromosomal translocations with deregulated BCL6 expression are frequently observed in diffuse large B cell lymphomas and follicular lymphomas, the two most common types of non–Hodgkin lymphoma (5, 6). The mechanism of how BCL6 acts as an oncogene has remained unclear. It has been thought that tumor-associated persistent expression of BCL6 at late stages of B-cell differentiation blocks terminal differentiation, increases cell survival, and progresses toward the transformed phenotype (7). Aberrant BCL6 expression may repress Blimp1, which induces B-cell differentiation into plasma cells and inhibits c-Myc expression, leading to failure in both B cell differentiation and cell cycle arrest (8). BCL6 also inhibits the expression of tumor suppressor p53, preventing p53-mediated cell cycle arrest and apoptotic response (9). Interestingly, BCL6 directly represses p21 expression through its binding to Miz-1 and suppresses cell cycle arrest in germinal center B cells (10). The repressive effect of BCL6 on these cell cycle regulators may partly account for the pathologic roles of BCL6 in lymphomagenesis.

Loss of the TGF-β antiproliferative response is a hallmark in human cancer (1, 11). However, genetic mutations in Smads or TGF-β receptors are rare in immune system–derived cancers (12). Thus, the mechanisms underlying TGF-β resistance in lymphoma or leukemia are largely elusive. In this study, we report a physical and functional interaction between BCL6 and Smad4. We found that B lymphoma cells that express a high level of BCL6 are refractory to TGF-β growth-inhibitory response, whereas knockdown of BCL6 expression restores TGF-β–mediated cell cycle arrest in B lymphoma cells. The results strongly suggest that aberrant expression of BCL6 promotes lymphomagenesis partly through its inhibition on TGF-β signaling.

Materials and Methods

Expression plasmids. The mammalian expression plasmids for Flag/HA-tagged Smads and rat TGF-β type I receptor (T202D) have been previously described (13). The BCL6 coding region was subcloned into pRK5 vector (Genentech) as Flag- or HA-tagged BCL6 proteins. Reporter SBE-Luc (14) was used to measure TGF-β/Smad-mediated transcriptional responses. The reporter 5xBCL6-Luc, containing five copies of BCL6 cognate binding sites, was used to detect transcriptional repression from BCL6 (15).

Cell culture, transfection, immunoprecipitation, and Western blotting. Human EBV-negative Burkitt-like lymphoma cell lines, BJAB, Ramos, Daudi, and Raji were provided by Paul Ling, and cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. BJAB cells were transiently transfected using Nucleofactor Kit T following the instructions of the manufacturer (Amaxa Biosystems).

Immunoprecipitation from 293T cell lysates using anti-Flag (M2) antibody were performed as described (13). Endogenous BCL6 in B lymphoma cells was immunoprecipitated by rabbit anti-BCL6 (N3, Santa Cruz Biotechnology). Immunoprecipitated proteins were analyzed by Western blotting using appropriate primary antibodies. The HDAC-Smad4-BCL6 ternary complex was detected as previously described (13).

Antibody-based array screening. The filter arrayed with antibodies (Hypromatrix) was incubated with the lysate of 293T cells transfected with Flag-Smad4 expression plasmid. The antibody-antigen-Smad4 complex was detected by anti-Flag antibody conjugated with horseradish peroxidase. Experimental details have been previously described (16).

shRNA-mediated knockdown of BCL6 expression. DNA coding for the target small interfering RNA sequence against BCL6 (9) was subcloned into retroviral vector pSRG (17). pSRG-shBCL6 was transfected into 293T with

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packaging vector pCL-Ampho. Forty-eight hours after transfection, virus-containing medium was harvested and passed through a 0.45-μm sterile filter. Exponentially growing BJAB cells were infected by the filtered medium supplemented with 0.5 μg/mL of polybrene. Forty-eight hours after infection, the medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum and 2 μg/mL of puromycin.

Growth inhibition assays. B lymphoma cells were seeded in 96-well (for MTT) or 12-well plates (for [^{3}H]thymidine) in appropriate density, and treated with or without TGF-β. The assays using MTT One Solution kit (Promega) were carried out as recommended by the manufacturer. DNA synthesis assay using [^{3}H]thymidine incorporation was performed as described (13).

Chromatin immunoprecipitation. BJAB cells (2×10^7) were cross-linked with formaldehyde, lysed, sonicated, and subjected to immunoprecipitation, essentially as described in an Upstate protocol. Immunoprecipitation was carried out using anti-Smad4 (B8), anti-BCL6 (N3), or control IgG serum, coupled with protein G-Sepharose beads. Immunocomplexes were reverse cross-linked to release DNA fragments, which were amplified by PCR with primers corresponding to PAI-1 promoter (138 bp). The PAI-1 promoter primer set contains 5′-atggtaacccctggtcccgttc-3′ and 5′-gatacacggctgactcccccac-3′. A sonication control PCR (260 bp) was done using primer set 5′-ttccattgctctaggatgcag-3′ and 5′-tgaatctgctgctgggtttct-3′.

Results

TGF-β resistance is associated with overexpression of BCL6 in B lymphoma cells. TGF-β inhibits lymphocyte proliferation (12). We first examined proliferation of several B lymphoma cell lines in response to TGF-β. As shown in Fig. 1*A*, BJAB, Loukes, Raji, and Daudi cells proliferated normally and exhibited a TGF-β–resistant phenotype, whereas BL41 showed sensitivity to TGF-β's antiproliferative effect. We then measured the levels of BCL6 in these B lymphoma cell lines, and found that BCL6 is highly expressed in all four TGF-β–resistant cell lines, but not in BL41 (Fig. 1*B*). This result indicates that the high level of BCL6 is closely correlated with TGF-β resistance in B lymphoma cells.

We also tested the effect of BCL6 on TGF-β–induced transcriptional activation. Cell cycle inhibitor p21 is a TGF-β signaling target gene, and its promoter is often used to faithfully reflect the growth-inhibitory effect of TGF-β. As shown in Fig. 1*C*, BCL6 dramatically represses TGF-β–induced p21 promoter activity.

Knockdown of BCL6 restores response to TGF-β signaling in B lymphoma cells. If the resistance to TGF-β–mediated growth arrest results from BCL6 overexpression, reducing BCL6 level in B lymphoma cells may restore their response to TGF-β–mediated growth arrest. Thus, we knocked down BCL6 expression in BJAB cells. Judging from anti-BCL6 Western blotting analysis, we observed a significant decrease (routinely ~60% knockdown) of BCL6 protein in BJAB cells infected with shBCL6 retroviruses, compared with those infected with control shRNA retroviruses (Fig. 2*A*). We also examined the effect of BLC6 on the activity of 5xBCL6-Luc reporter, the transcription of

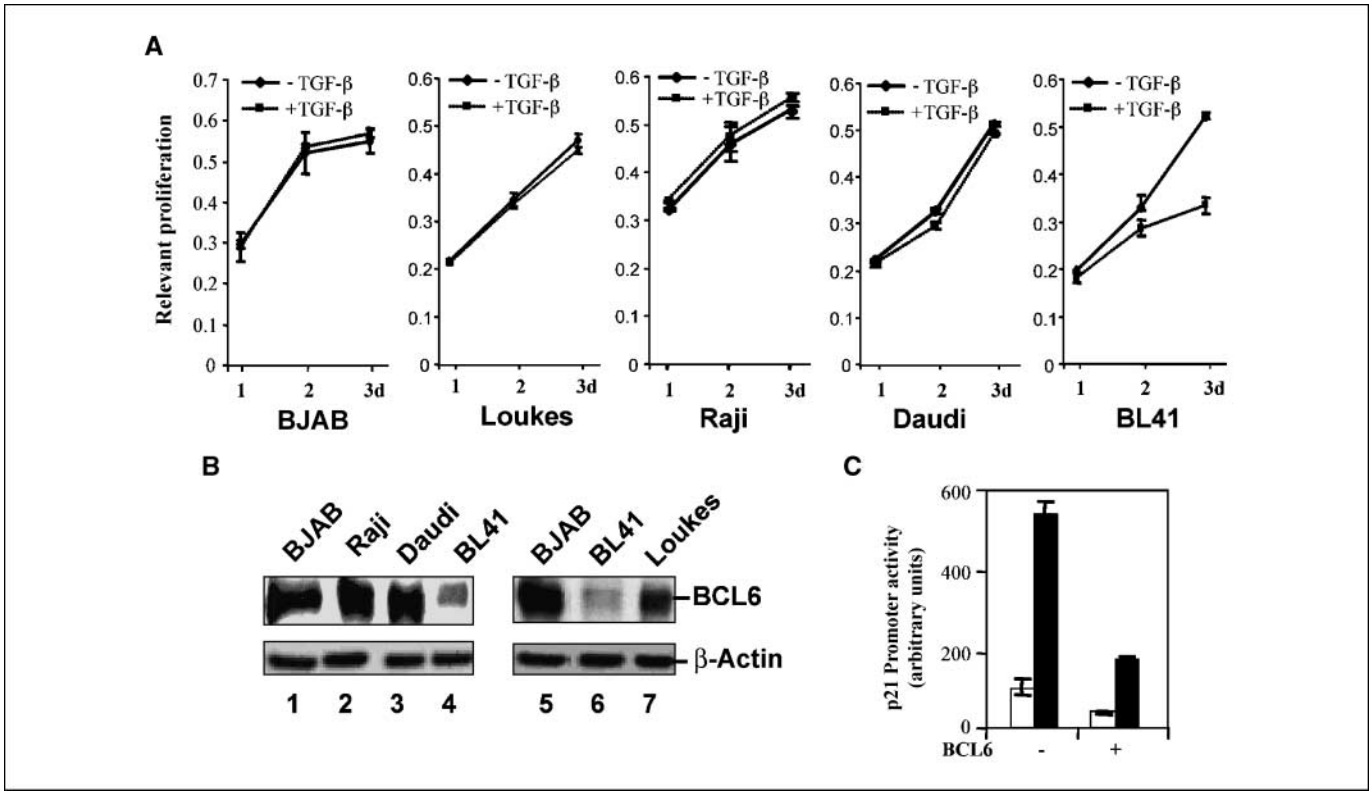

Figure 1. Correlation between BCL6 and TGF-β resistance in B lymphoma cells. *A,* responsiveness to TGF-β–induced growth inhibition in various B lymphoma cells. B lymphoma cells were seeded in 96-well plates and treated with or without 10 ng/mL of TGF-β. Cells were harvested at 1, 2, and 3 d after adding TGF-β, followed by growth measurement using the MTT kit. *B,* BCL6 expression level in various B lymphoma cells. B lymphoma cells showing resistance to TGF-β–induced growth inhibition have high levels of BCL6 protein, whereas BL41 cells with responsiveness to TGF-β–induced growth inhibition show a low level of BCL6 protein. β-Actin levels indicate equal loading of proteins from various B lymphoma cells. *C,* repression of BCL6 on p21-Luc transactivation stimulated by TGF-β. $HepG_2$ cells were cotransfected with p21-Luc and the expression plasmid of BCL6. Twelve hours after transfection, cells were treated with 400 pmol/L of TGF-β for 24 h, followed by cell harvest and luciferase activity measurement.

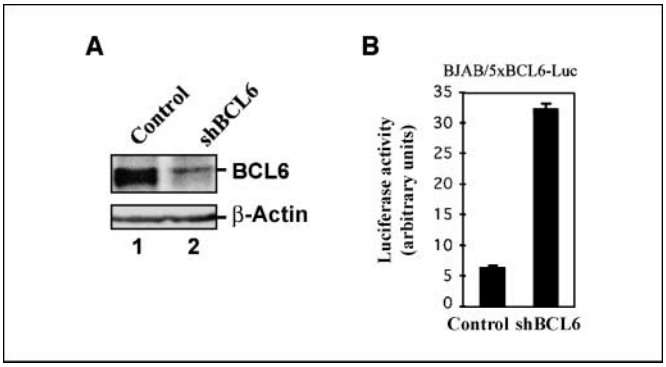

Figure 2. shRNA-mediated knockdown of BCL6. *A,* knockdown of BCL6 expression in BJAB cells. BJAB cells were infected by control shRNA or BCL6 shRNA retroviruses and subsequently lysed for anti-BCL6 Western blotting assay. β-Actin levels indicate equal sample loading. *B,* 5xBCL6-Luc reporter activity was increased in BJAB cells infected by BCL6 shRNA retrovirus. Expression plasmid of 5xBCL6-Luc reporter was transfected into BJAB cells expressing control or BCL6 shRNA using AMAXA.

which could be repressed by BCL6 (15). Higher 5xBCL6-Luc activity was observed in BJAB cells expressing shBCL6 compared with controls (Fig. 2*B*), thereby functionally confirming the knockdown of BCL6 in shBCL6 cells.

We next investigated whether BCL6 knockdown affected TGF-β–induced transcription. As shown in Fig. 3*A*, there was no obvious increase of SBE-Luc transcription upon TGF-β stimulation in BJAB cells expressing control shRNA. However, SBE-Luc activity was induced by TGF-β in cells expressing shBCL6, indicating that reduced expression of BCL6 in B lymphoma cells restores the TGF-β–induced SBE-Luc response.

Finally, we determined whether TGF-β–mediated growth arrest was restored in BJAB cells expressing shBCL6. As shown in Fig. 3*B*, BJAB cells expressing control shRNA did not show any change in growth upon TGF-β treatment. In contrast, BJAB cells expressing shBCL6 proliferated more slowly, and more importantly, TGF-β inhibited DNA synthesis in the shBCL6 cells in a dose-dependent manner (Fig. 3*B*). In accordance, Western blotting analysis indicates that TGF-β induced significantly higher levels of all TGF-β target genes tested including p21, p15, and PAI-1 (Fig. 3*C*). We also noted that basal levels of p21 increased in BCL6 knockdown BJAB cells, suggesting that depletion of BCL6 may also enhance the activity of endogenous p53 (9, 10) as well as that of autocrine TGF-β. This result clearly shows that reduced BCL6 expression renders BJAB cells sensitive to TGF-β–mediated growth inhibition, supporting the notion that high BCL6 levels in B lymphoma cells inhibits TGF-β–mediated growth inhibition. Furthermore, BCL6 knockdown also sensitized BJAB cells to exhibit higher induction of Id1 reporter expression in response to BMP2 (Fig. 3*D*).

BCL6 interacts with Smad4 under physiologic conditions. In a parallel attempt to identify regulators of TGF-β signaling, we launched an antibody array–based screen to search for novel Smad4-associated cellular proteins. Smad4 is the central mediator in TGF-β signaling and is frequently deleted or mutated in cancers (11). Among 400 signal transducers or transcription factors, 20 proteins interacted with Smad4 (data not shown). BCL6 was also identified as a candidate Smad4-

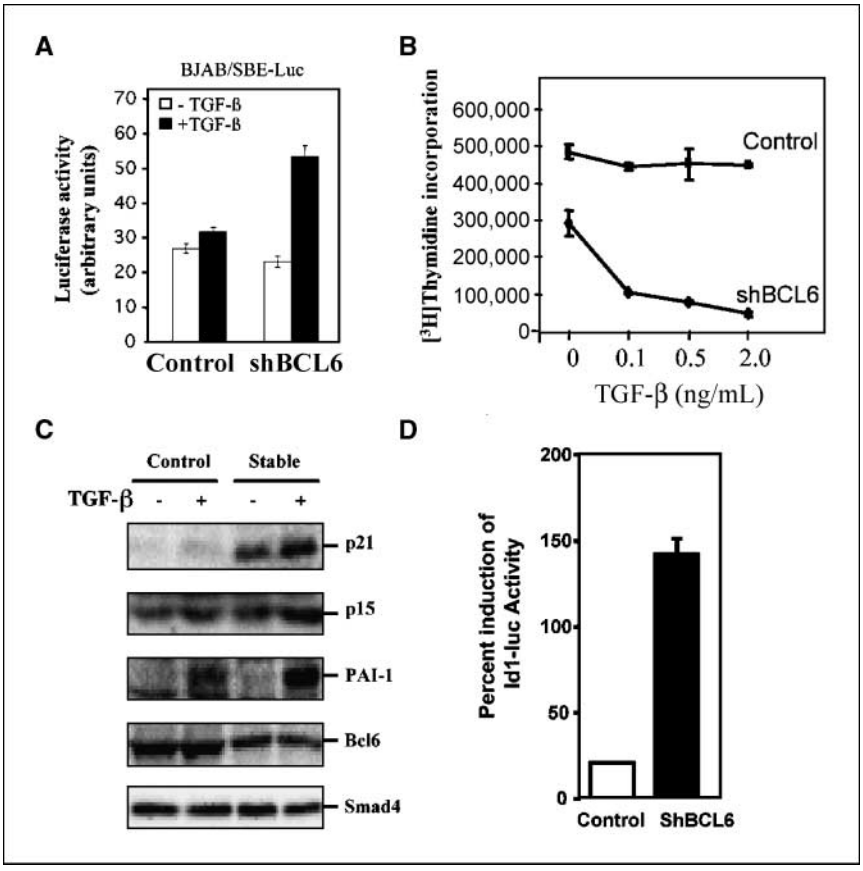

Figure 3. Knockdown of BCL6 by shRNA restores sensitivity to TGF-β in B lymphoma cells. *A,* knockdown of BCL6 expression restores TGF-β–mediated SBE-luc transcription in BJAB cells. BJAB cells expressing control or BCL6 shRNA were transfected with SBE-Luc expression plasmid and treated with TGF-β for 24 h before transcription assay. *B,* knockdown of BCL6 expression restores TGF-β–mediated growth arrest in BJAB cells. Cells were treated with increasing concentrations of TGF-β for 48 h before adding [^{3}H]thymidine. Four hours after adding [^{3}H]thymidine, cells were harvested and subjected to treatment with trichloroacetic acid, followed by scintillation assay. *C,* knockdown of BCL6 expression restores TGF-β–induced gene expression in BJAB cells. shBCL6 and control cells were treated with 2 ng/mL of TGF-β for 48 h, and lysates were subjected to Western blotting analysis with antibodies against the indicated proteins. *D,* shBCL6 sensitizes BJAB cells to respond to BMP2 to induce Id1-Luc expression. BJAB cells were cotransfected with Id1-Luc and the expression plasmid of BCL6. Twelve hours after transfection, cells were treated with 25 ng/mL of BMP2 for 24 h, followed by cell harvest and luciferase activity measurement.

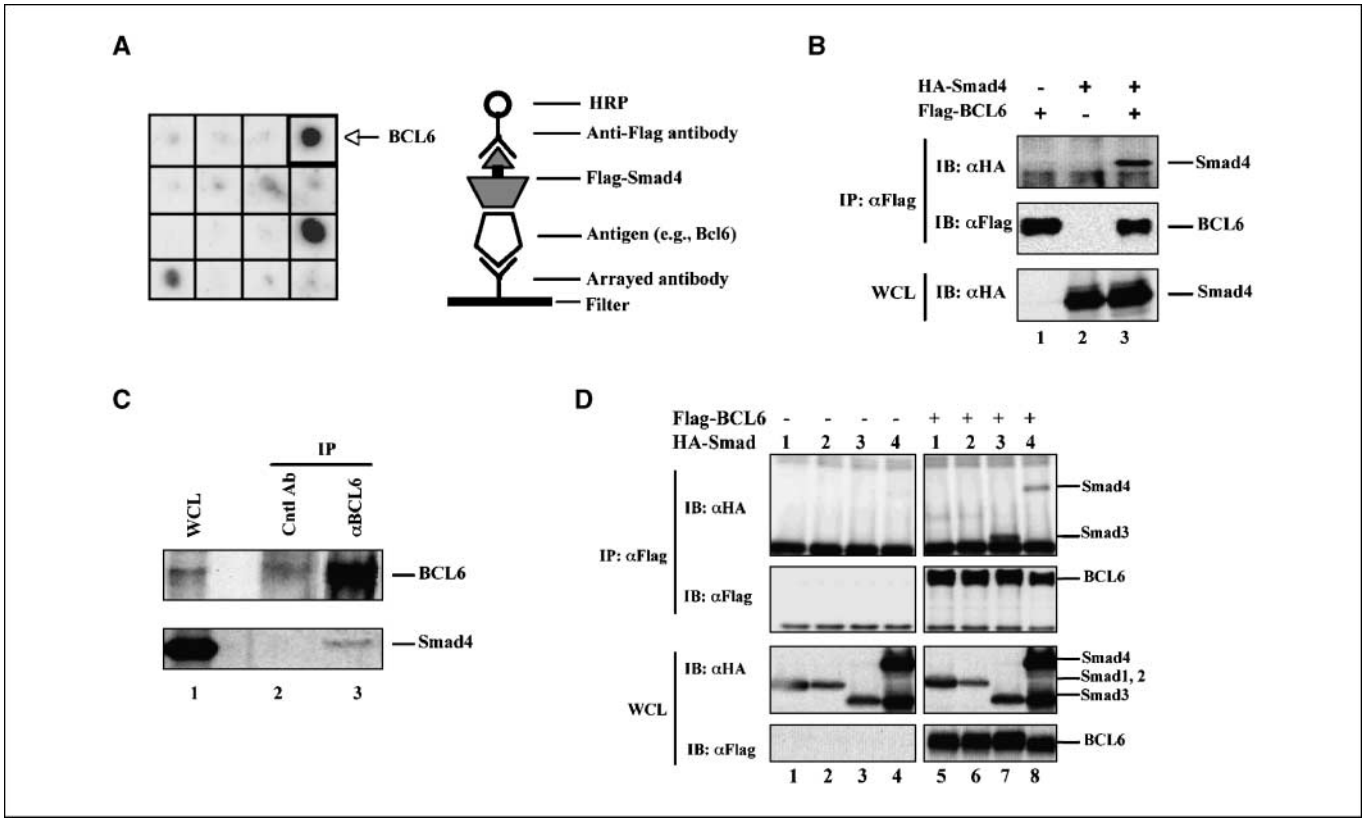

Figure 4. BCL6 physically interacts with Smad4. *A,* identification of BCL6 as Smad4-interacting protein by antibody array–based screening. A nitrocellulose filter arrayed with antibodies against 400 signal transduction proteins were blotted with lysates of 293T cells transfected with Flag-tagged Smad4. The multiprotein complex composed of the arrayed antibody, its antigen (potential Smad4-binding protein), and Smad4 was detected by immunoblotting with a horseradish peroxidase–conjugated anti-Flag antibody and visualized by chemiluminescence. *Right,* part of the filter containing the antibody against BCL6. *B,* BCL6 interacts with Smad4 *in vivo*. 293T cells were transfected with Flag-tagged BCL6 and HA-tagged Smad4. Cell lysates were immunoprecipitated (*IP*) with anti-Flag antibody, followed by immunoblotting (*IB*) with an anti-HA antibody to detect BCL6-bound Smad4 (*top*) and anti-Flag to the level of immunoprecipitated BCL6 (*middle*). Whole cell lysates (*WCL*) were also directly immunoblotted with anti-HA antibody to show the expression of transfected Smad4 (*bottom*). *C,* endogenous interaction between BCL6 and Smad4. Exponentially growing BJAB cells were subjected to immunoprecipitation (*IP*) with polyclonal antibodies against BCL6 (N3, Santa Cruz Biotechnology) or a control antibody. BCL6-bound Smad4 was detected by immunoblotting (*IB*) with anti-Smad4 (*B8*). Levels of BCL6 in the immunoprecipitate and whole cell lysate (*WCL*) were also shown. *D,* BCL6 interacts with Smad3. 293T cells were transfected with Flag-tagged BCL6 and HA-tagged Smads. Cell lysates were immunoprecipitated (*IP*) with anti-Flag antibody, followed by immunoblotting (*IB*) with an anti-HA antibody to detect BCL6-bound Smads (*top*) and anti-Flag to the level of immunoprecipitated BCL6 (*top middle*). Whole cell lysates (*WCL*) were also directly immunoblotted with anti-HA and anti-Flag antibody to show the expression of transfected Smads (*bottom middle*) and BCL6 (*bottom*), respectively.

interacting protein (Fig. 4*A*). To further confirm this physical interaction between BCL6 and Smad4, we performed coimmunoprecipitation experiments. Flag-BCL6 and HA-Smad4 were cotransfected into 293T cells. Flag-BCL6 in cell lysates was immunoprecipitated with anti-Flag antibody, and the immunoprecipitated complex was subjected to Western blot analysis with anti-HA antibody to detect BCL6-bound Smad4. As shown in Fig. 4*B*, Smad4 was clearly coimmunoprecipitated with BCL6 when Smad4 and BCL6 were coexpressed in cells (*lane 3*).

To determine if BCL6 interacts with Smad4 at endogenous levels, anti-BCL6 antibody was used to immunoprecipitate endogenous BCL6 proteins in BJAB lymphoma cells and then the immunoprecipitated products were analyzed by anti-Smad4 Western blotting to detect BCL6-bound Smad4 protein. We found that Smad4 could be coimmunoprecipitated with BCL6 by anti-BCL6 antibody, but not by control antibody (Fig. 4*C, lanes 2* and *3*). Our finding indicated that Smad4 interacts with BCL6 under physiologic conditions.

To test if BCL6 interacts with other Smads, we performed coimmunoprecipitation experiments. Results shown in Fig. 4*D* confirm that Smad4 (*lane 8*) interacted with BCL6, and further showed that Smad3 (*lane 7*) was also clearly coimmunoprecipitated with BCL6. These results suggest that BCL6 interacts with both Smad3 and Smad4.

BCL6 does not interfere with Smad activation. Smad4 is the central mediator for TGF-β signaling. Upon TGF-β ligand binding, Smad4 forms a complex with Smad2/3, which translocates into the nucleus, where they cooperate with specific transcription factors or general transcriptional coactivators to exert their transcriptional regulation. After having determined that BCL6 interacts with Smad4, we further examined if BCL6 interferes with Smad phosphorylation and Smad2-Smad4 complex formation. As shown in Fig. 5*A*, BJAB cells, which express a high level of BCL6, retain the ability to induce endogenous Smad2/3 phosphorylation in response to TGF-β. In addition, BCL6 also had no effect on the phosphorylation of exogenous Smad2/3 induced by constitutively active TGF-β type I receptor (T202D; Fig. 5*B, lanes 4* and *8*), consistent with the ability of TGF-β to induce endogenous Smad2/3 phosphorylation in high BCL6-expressing BJAB cells (Fig. 5*A*). Although BCL6 interacted with Smad4, it did not interfere with the complex formation between Smad4 and Smad2/3, as Myc-Smad2/3 and Flag-Smad4 could be similarly coimmunoprecipitated in the presence or absence of BCL6 (Fig. 5*C*).

BCL6 disrupts the interaction between Smads and p300. Several studies have shown that transcriptional activity of Smads requires the involvement of coactivator p300/CBP (18–20). We next examined whether BCL6 interfered with the recruitment of p300/CBP to the Smad complex. Complex formation between p300 and Smad3/4 was investigated by coimmunoprecipitation experiments. Coexpression of constitutively active TGF-β type I receptor (T202D) induced a strong interaction between p300 and Smad4 (Fig. 6*A, lanes 3* and *4*) and between p300 and Smad3 (Fig. 6*B, lanes 3* and *4*). Importantly, BCL6 clearly attenuated the p300-Smad4 (Fig. 6*A, lane 5*) and p300-Smad3 interaction (Fig. 6*B, lane 5*). These results suggest that BCL6 negatively regulates the complex formation between Smad4 and coactivators, which in turn, represses Smad4-mediated transcription activation and TGF-β signaling.

Smads recruits BCL6 and class II HDACs into chromatin. To examine if BCL6 could be recruited to endogenous TGF-β–responsive promoters, we performed a chromatin immunoprecipitation assay on the PAI-1 promoter in BJAB cells. As expected, Smad4 bound to the promoter region (nucleotides −792/−655) of PAI-1 (Fig. 6*C, top gel panel, lane 3*). Notably, we found that BCL6 could also be recruited to the same PAI-1 promoter region (Fig. 6*C, top gel panel, lane 4*). Thus, BCL6 could bind to an endogenous TGF-β–responsive promoter in a chromatin environment. Furthermore, the binding of BCL6 and Smad4 in the PAI-1 promoter region was specific as both proteins failed to bind to a downstream region (nucleotides +1282/1542) of the PAI-1 promoter (Fig. 6*C, bottom gel panel, lanes 3* and *4*).

The COOH-terminal zinc finger domain of BCL6 binds to Smad4 and suffices to repress TGF-β signaling (data not shown). This repressive effect is achieved partly through the recruitment of class II histone deacetylases such as HDAC5 (21). To determine whether Smad4 interacts with BCL6 to recruit HDAC4/5, we examined the potential formation of a ternary complex consisting of Smad4, BCL6, and HDACs. We transfected Myc-BCL6 and HA-Smad4 together with Flag-HDAC4 or Flag-HDAC5 into 293T cells, constitutively active TGF-β type I receptor (T202D) was cotransfected to explore the effect of TGF-β treatment. As shown in Fig. 6*D*, only when the three components (BCL6, Smad4, and HDACs) were coexpressed did we detect a specific ternary complex. Furthermore, compared with HDAC4, HDAC5 shows a higher ability to form ternary complexes with Smad4 and BCL6 (Fig. 6*D, lanes 8* and *9* versus *lanes 4* and *5*), which is consistent with the higher affinity of HDAC5 to BCL6 (21). TGF-β treatment seems to decrease the HDAC4 ternary complex while not affecting the HDAC5 ternary complex (Fig. 6*D, lanes 5* and *9* versus *lanes 4* and *8*). These data suggest that through the interaction with Smad4, BCL6 could also recruit class II HDAC into the Smad complex, providing another mechanism to repress TGF-β signaling.

Discussion

BCL6 is frequently overexpressed in non–Hodgkin lymphomas (6). The oncogenic role of BCL6 was also defined in engineered mice expressing BCL6 constitutively, in which B cell lymphoma was exhibited at high penetrance (22). Although it is generally thought that BCL6 exerts its effect on lymphomagenesis through transcriptional repression of target genes that control B cell activation and differentiation (5, 6), precise mechanisms for how BCL6 contributes to lymphomagenesis remain unclear. Recently, it has been reported that BCL6 represses the transcription of *p53*, thus inhibiting p53-dependent cell growth arrest and apoptosis (9). In addition, BCL6 also inhibits cell growth arrest by repressing p21 promoter via binding to Miz-1 (10). Our current study identifies Smad4 as another tumor suppressor that BCL6 antagonizes, suggesting that targeting tumor suppressor pathways may be one general mechanism underlying BCL6's oncogenic functions.

Loss of TGF-β–mediated growth control represents one major mechanism in cancer development (11). In addition to genetic lesions in the genes encoding Smads and TGF-β receptors, accumulating evidence has shown that the tumor suppressor functions of Smads are blocked by oncogene products such as c-Ski, c-Myc, and Evi-1 through direct Smad-oncoprotein interactions (2). As B lymphomas often overexpress oncogene BCL6, we reasoned that BCL6 might contribute to TGF-β

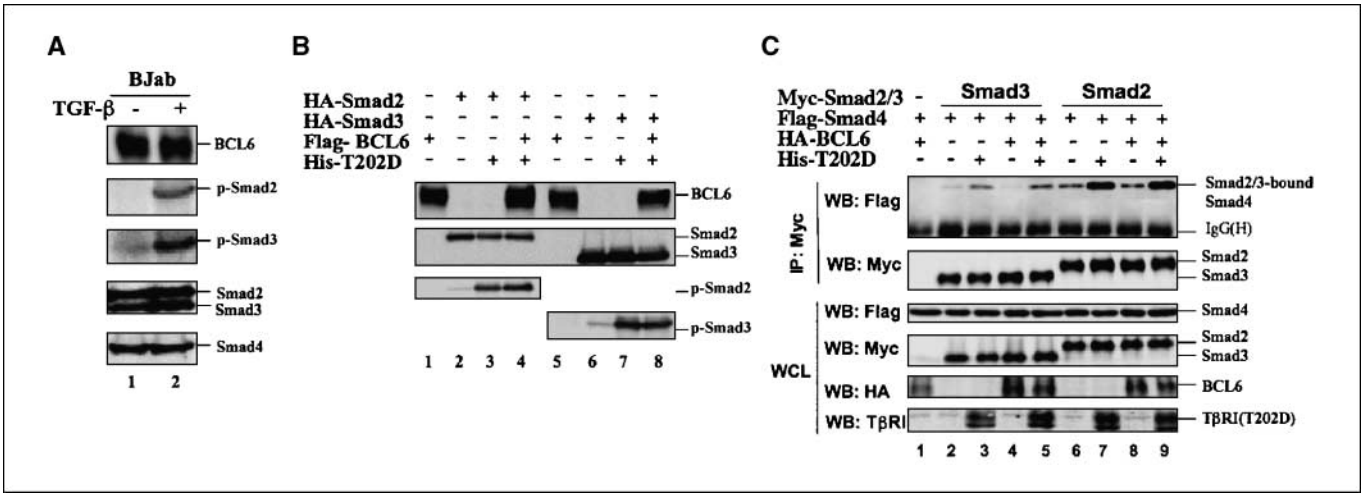

Figure 5. BCL6 has no effects on Smad activation. *A*, endogenous phosphorylation of Smad2 and Smad3 in BJAB cells upon TGF-β treatment. BJAB cells were treated with or without TGF-β for 2 h. Endogenous levels of Smad2/3, P-Smad2/3, and Smad4 were detected with corresponding antibodies. *B,* BCL6 has no effects on the phosphorylation of Smad2 and Smad3. Flag-BCL6 and HA-Smad2 or HA-Smad3 were cotransfected with or without His-T202D (constitutively active TGF-β type I receptor) into 293T cells. Phosphorylated Smad2 and Smad3 were detected in the Western blot using appropriate antibodies. *C,* BCL6 does not alter the Smad complex formation. Combinations of expression plasmids as indicated were cotransfected into 293T. The levels of Smad2/3-bound Smad4 did not change in the presence of BCL6 in comparison with that in the absence of BCL6.

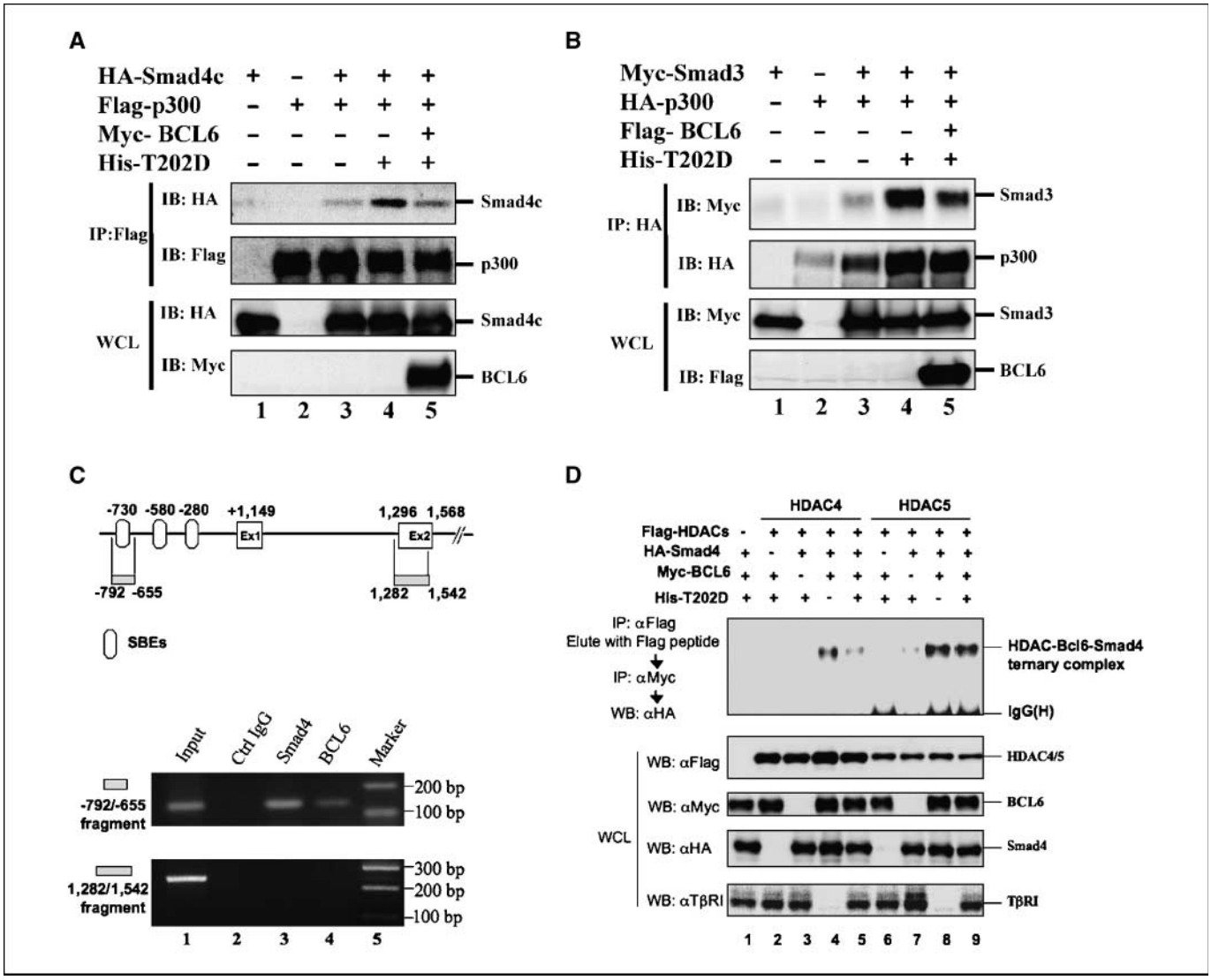

Figure 6. BCL6 alters the Smad-chromatin relationship. *A,* BCL6 attenuates the association between Smad4 and p300. HA-Smad4c and Flag-p300 were cotransfected into 293T cells together with Myc-BCL6 in the presence or absence of His-T202D. 293T cell lysates were immunoprecipitated with anti-Flag antibody. p300-bound Smad4 were detected with anti-HA. Immunoprecipitated Flag-p300 and protein expression levels in whole cell lysates were blotted with appropriate antibodies. *B,* BCL6 attenuates the association between Smad3 and p300. Myc-Smad3 and HA-p300 were cotransfected into 293T cells together with Flag-BCL6 in the presence or absence of His-T202D. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. *C,* BCL6 associates with Smad4 on the PAI-1 promoter. Chromatin DNA from BJAB cells were immunoprecipitated with control IgG, anti-Smad4, or anti-BCL6 antibodies. PCR was performed using DNA fragments extracted from immune complexes as templates. The 138 bp PCR product spanning a Smad-binding element (*SBE*) showed BCL6 recruitment to the PAI-1 promoter region. A 260 bp fragment in exon 2 was included for a chromatin immunoprecipitation control. *Top,* a schematic diagram for the PAI-1 promoter. *D,* Smad4, BCL6, and class II HDACs could form a ternary complex. 293T cells were transfected with combinations of plasmids as indicated. Cell lysates were immunoprecipitated (*IP*) with anti-Flag agarose beads, eluted with Flag peptide, followed by a second immunoprecipitation with anti-Myc agarose beads. The ternary complex was detected by anti-HA immunoblot. Protein expression level in whole cell lysates (*WCL*) was shown by immunoblots with appropriate antibodies.

resistance in B lymphoma cells. Indeed, our current study reveals a novel function of BCL6. Its high expression level inhibits the tumor-suppressive function of Smads. Notably, shRNA-mediated knockdown of BCL6 expression sensitizes B lymphoma cells to TGF-β–mediated antiproliferative effects. BCL6 does not affect TGF-β–induced signaling events in the cytoplasm such as Smad2/3 phosphorylation and formation of the Smad2/3-Smad4 complex. Consistent with its nuclear repressor function, BCL6 dampens the interaction between Smads and coactivator p300, and at the same time recruits HDAC4 and HDAC5 into the complex to ensure complete blockade of TGF-β signaling. In conclusion, our study provides compelling evidence demonstrating that BCL6 is a new oncogenic partner that interacts with Smads and suggests that BCL6 induces lymphomagenesis partly through disruption of the TGF-β pathway.

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References

1. ten Dijke P, Heldin CH. Smad signal transduction. Dordrecht: Springer; 2006. vol. 5. p. 1–472.
2. Feng X-H, Derynck R. Specificity and versatility in TGF-β signaling through Smads. Ann Rev Cell Dev Biol 2005;21:659–93.
3. Massague J, Seoane J, Wotton D. Smad transcription factors. Genes Dev 2005;19:2783–810.
4. Ye BH, Lista F, Lo Coco F, et al. Alterations of a zinc finger-encoding gene, BCL-6, in diffuse large-cell lymphoma. Science 1993;262:747–50.
5. Ohno H. Pathogenetic role of BCL6 translocation in B-cell non-Hodgkin's lymphoma. Histol Histopathol 2004;19:637–50.
6. Pasqualucci L, Bereschenko O, Niu H, et al. Molecular pathogenesis of non-Hodgkin's lymphoma: the role of Bcl-6. Leuk Lymphoma 2003;44 Suppl 3: S5–12.
7. Lossos IS, Akasaka T, Martinez-Climent JA, Siebert R, Levy R. The BCL6 gene in B-cell lymphomas with 3q27 translocations is expressed mainly from the rearranged allele irrespective of the partner gene. Leukemia 2003;17: 1390–7.
8. Shaffer AL, Lin KI, Kuo TC, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. Immunity 2002;17:51–62.
9. Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. Nature 2004;432:635–9.
10. Phan RT, Saito M, Basso K, Niu H, Dalla-Favera R. BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. Nat Immunol 2005;6:1054–60.
11. Levy L, Hill CS. Alterations in components of the TGF-β superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev 2006;17:41–58.
12. Kim SJ, Letterio J. Transforming growth factor-β signaling in normal and malignant hematopoiesis. Leukemia 2003;17:1731–7.
13. Feng X-H, Liang Y-Y, Liang M, Zhai W, Lin X. Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-β-mediated induction of the CDK inhibitor p15INK4B. Mol Cells 2002;9:133–43.
14. Zawel L, Dai JL, Buckhaults P, et al. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cells 1998;1:611–7.
15. Huynh KD, Fischle W, Verdin E, Bardwell VJ. BCoR, a novel corepressor involved in BCL-6 repression. Genes Dev 2000;14:1810–23.
16. Liang M, Liang YY, Wrighton K, et al. Ubiquitination and proteolysis of cancer-derived Smad4 mutants by SCFSkp2. Mol Cell Biol 2004;24:7524–37.
17. Lin X, Duan X, Liang YY, et al. PPM1A functions as a Smad phosphatase to terminate TGFβ signaling. Cell 2006;125:915–28.
18. Feng X-H, Zhang Y, Wu R-Y, Derynck R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for Smad3 in TGF-β-induced transcriptional activation. Genes Dev 1998; 12:2153–63.
19. Janknecht R, Wells NJ, Hunter T. TGF-β-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev 1998;12:2114–9.
20. de Caestecker MP, Yahata T, Wang D, et al. The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. J Biol Chem 2000;275:2115–22.
21. Lemercier C, Brocard MP, Puvion-Dutilleul F, Kao HY, Albagli O, Khochbin S. Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor. J Biol Chem 2002;277:22045–52.
22. Cattoretti G, Pasqualucci L, Ballon G, et al. Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. Cancer Cell 2005;7:445–55.