BCL9 Promotes Tumor Progression by Conferring Enhanced Proliferative, Metastatic, and Angiogenic Properties to Cancer Cells

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Abstract

Several components of the Wnt signaling cascade have been shown to function either as tumor suppressor proteins or as oncogenes in multiple human cancers, underscoring the relevance of this pathway in oncogenesis and the need for further investigation of Wnt signaling components as potential targets for cancer therapy. Here, using expression profiling analysis as well as *in vitro* and *in vivo* functional studies, we show that the Wnt pathway component BCL9 is a novel oncogene that is aberrantly expressed in human multiple myeloma as well as colon carcinoma. We show that BCL9 enhances β-catenin–mediated transcriptional activity regardless of the mutational status of the Wnt signaling components and increases cell proliferation, migration, invasion, and the metastatic potential of tumor cells by promoting loss of epithelial and gain of mesenchymal-like phenotype. Most importantly, BCL9 knockdown significantly increased the survival of xenograft mouse models of cancer by reducing tumor load, metastasis, and host angiogenesis through down-regulation of c-Myc, cyclin D1, CD44, and vascular endothelial growth factor expression by tumor cells. Together, these findings suggest that deregulation of BCL9 is an important contributing factor to tumor progression. The pleiotropic roles of BCL9 reported in this study underscore its value as a drug target for therapeutic intervention in several malignancies associated with aberrant Wnt signaling. [Cancer Res 2009;69(19):7577–86]

Introduction

The Wnt pathway consists of a highly conserved and tightly regulated receptor-mediated signal transduction system (1–3). Association of Wnt ligands with Frizzled and low-density lipoprotein receptor–related protein (LRP5 and LRP6) coreceptors leads to stabilization of β-catenin, a key event in this signal transduction pathway. In the absence of Wnt signal, β-catenin is phosphorylated by a destruction complex consisting of adenomatous polyposis coli, Axin, glycogen synthase kinase-3β, and casein kinase 1α, marking it for ubiquitination and proteasome degradation. In the presence of Wnt ligands, this destruction complex is dissociated and the unphosphorylated active β-catenin translocates to the nucleus, where it functions as a transcriptional activator to activate the expression of target genes, such as c-Myc and cyclin D1 (4, 5).

Dysregulation of the canonical Wnt/β-catenin pathway has been implicated in numerous human malignancies, generating immense interest in these molecules as targets for cancer therapy (6). The molecular genetics underlying Wnt/β-catenin activation in cancer centers on mutations that enable β-catenin to escape the destruction complex and accumulate in the nucleus. Loss-of-function mutations in the tumor suppressors adenomatous polyposis coli and Axin, as well as activating mutations in β-catenin, have been identified in several human cancers including colon carcinomas (6, 7). In addition to these defined mutations, accumulation of nuclear β-catenin has been shown in multiple myeloma, which lacks known mutations of the Wnt pathway genes, indicating alternate pathways for β-catenin activation (8–10). Two recently identified nuclear Wnt pathway components, BCL9 and Pygopus, have been shown to play an important role in transcriptional activity of β-catenin in association with LEF/TCF family members (11). The *BCL9* gene resides on chromosome 1q21, a region frequently involved in secondary chromosomal abnormalities and associated with poor clinical outcome as well as overexpression of resident genes (12, 13). *BCL9* was identified as a gene overexpressed in a B-cell acute lymphoblastic leukemia cell line with t(1;14)(q21;q32) translocation (14). In addition, BCL9 has recently been shown to possess a potent transcription activation domain (15, 16). Taken together, these findings suggest that BCL9 might behave as an oncogene by providing an alternate pathway for β-catenin activation and subsequent tumor progression.

Here, we provide evidence that BCL9 deregulation is one such alternate mechanism of activation. Regardless of the mutational status of the Wnt signaling components, BCL9 enhanced β-catenin–mediated transcriptional activity in cancers with (colon carcinoma) and without (multiple myeloma) Wnt/β-catenin activating mutations. BCL9 is overexpressed in a subset of multiple myeloma and colon carcinoma primary tumors as well as cell lines. BCL9 enhanced proliferation, survival, migration, invasion, and the metastatic potential of tumor cells. In addition, BCL9 knockdown enhanced the survival of xenograft mouse models of cancer and attenuated the expression of CD44 and vascular endothelial growth factor (VEGF) secretion by tumor cells, thereby reducing tumor metastasis and host angiogenesis. We provide evidence for the involvement of BCL9 in the progression of these malignancies by favoring an epithelial-to-mesenchymal transition (EMT)–like differentiation of tumor cells. These data confirm that BCL9 is involved in tumor progression, underscoring

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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its relevance as a novel therapeutic target in cancers associated with dysregulated Wnt activity.

Materials and Methods

Cell lines. Plasma cells and multiple myeloma cell lines were collected and maintained as described (8). Colon cancer cell lines were obtained from the American Type Culture Collection and the German Collection of Microorganisms and Cell Cultures. Control and Wnt3A conditional media were collected from supernatants of L cells (CRL-2648) and L-Wnt3A cells (CRL-2647) were from the American Type Culture Collection.

Expression profiling. Affymetrix GeneChip for multiple myeloma primary tumors, cell lines, and normal plasma cells was obtained from the National Center for Biotechnology Information Gene Expression Omnibus.[8] RNA from colon carcinoma primary tumors, cell lines, and normal cells were run on Affymetrix Human Exon 1.0 ST Array. RNA from MM1S and Colo320 control shRNA or BCL9 shRNA cells were run on an Affymetrix U133A 2.0 array chip as described (8). Statistical analyses were done in R[9] using Bioconductor software (17). Probe-set expression values were generated using "plier."[10] Moderated t test was applied and a P-value cutoff of 0.05 after multiple testing adjustments with BH method was used (18).

Lentiviral production. The BCL9 cDNA that had an extra base pair at position 4,171 was removed by QuikChange site-directed mutagenesis kit (Stratagene). The resulting BCL9 cDNA was similar to the one described (14) and used for further studies. FLAG-tagged BCL9 cDNA was cloned into lentiviral pHAGECMV/UBC-green fluorescent protein (GFP) vector. Lentiviral particles were generated in 293T cells as described (8). Two lentiviral pLKO.1 shRNA-based BCL9 constructs, shRNA1 (gctgcaatacacagtgttatt) and shRNA2 (cctctgtgaatatccctggaa), showed efficient knockdown in several cell lines and BCL9 shRNA2 was used for further studies. A puromycin selection marker from the pLKO.1 shRNA vector was replaced by GFP and GFP-positive cells were sorted 4 days after infection. All assays were all done with transduced cells that were passaged no more than two times.

Cell proliferation and colony formation assays. Cell proliferation by [^{3}H]thymidine uptake was measured as described (8). Soft-agar colony assays were done in RPMI 1640-10% fetal bovine serum as described (19). Briefly, six-well plates (Falcon) were covered with 2 mL of 0.8% SeaPlaque agarose (FMC) and allowed to solidify at room temperature. MM1S and Colo320 cells (500 cells in 200 μL/well) were mixed with 1 mL of 0.5% SeaPlaque agarose and added on top of the base agar. Colonies were stained with *p*-iodonitrotetrazolium violet (50 mg/mL; Sigma) and counted after 15 days. Experiments were done in triplicate and repeated twice.

Migration and invasion assays. For migration, the top chamber of Transwell plates (Corning Costar) were seeded with 2×10^5 multiple myeloma cells in triplicates and control L-medium or Wnt3A-containing medium was added to the bottom chamber. Cells in the bottom wells were collected and counted after 10 h incubation. For invasion, 2×10^5 cells per well were resuspended in serum-free RPMI 1640 and seeded in Matrigel Boyden chambers (BD Biosciences) in triplicate with 10% serum-containing medium as a chemoattractant as well as in regular cell culture plates as input controls. Following 22 h incubation, cells that migrated through the chamber were fixed in methanol, stained with crystal violet, and counted. Experiments were repeated twice.

Antibody generation. A COOH-terminal *Sma*I-*Sma*I fragment of BCL9 cDNA corresponding to amino acids 1,198 to 1,404 of human BCL9 was generated as a GST-fusion protein, purified on a GST column (New England Biolabs), and injected into rabbit to generate polyclonal antibody sera 6109. Affinity purification was done using an activated agarose matrix (Rockland). Affinity-purified BCL9 antibody was tested on the cell lysates overexpressing FLAG-BCL9 and verified with FLAG-M2 antibody (Sigma).

Quantitative reverse transcription-PCR. RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis was done with 2 μg RNA for each sample using SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Quantitative reverse transcription-PCR was carried out using Eppendorf Mastercycler realplex2 and analysis of target genes was done in triplicates using the SYBR Green quantitative PCR master mix (Fermentas) with the designated forward and reverse primers: BCL9 (CCAACTTGCCATCAATGAA-TAA and GGCATCTGATTGGAGTGAGAA), c-Myc (TTTTTCGGGTAGTG-GAAAACC and GCAGTAGAAATACGGCTGCAC), cyclin D1 (5TGTCCTACTA-CCGCCTCACAC and TGGGGTCCATGTTCTGCT), CD44 (TTTGCATTGCA-GTCAACAGTC and TGAGTCCACTTGGCTTTCTGT), and VEGF (CAT-GAACTTTCTGCTGTCTTGG and ATGATTCTGCCCTCCTCCTT). Gene expression values were calculated relative to β-actin expression for each sample.

Immunoblotting analysis. Immunoblot analysis was done as described previously (8) using antibodies against BCL9 (6109), β-catenin (CAT5-H10; Zymed), c-Myc (OP10; Calbiochem), BCL9 (ab37305; Abcam), BCL9 peptide (ab37304; Abcam), glyceraldehyde-3-phosphate dehydrogenase (ab9485; Abcam), and Igλ (DB2001; DeltaBiolabs). Antibodies against GFP (B-2), FLAG-M2, actin-horseradish peroxidase (C-11), and cyclin D1 (H-295) and secondary antibodies conjugated to horseradish peroxidase were from Santa Cruz Biotechnology and Southern Biotechnology.

Immunofluorescence analysis. Cell samples were prepared using a cytocentrifuge (Thermo Shandon; ref. 8) or grown on polylysine-coated slides. Cells were fixed 20 min at room temperature in 2% paraformaldehyde, permeabilized in TBS-Tween 20 for 20 min, washed three times in PBS, and blocked with 5% bovine serum albumin in PBS for 2 h before addition of primary antibodies. Immunofluorescence analysis for BCL9 expression was done using the commercially available anti-BCL9 (ab37305; Abcam). Anti-β-catenin (CAT5-H10; Zymed), vimentin (Vim 3B4; DAKO), E-cadherin (E10; Cell Signaling), and BCL9 (ab37305; Abcam) antibodies were added overnight at 4°C and washed in PBS. Secondary antibodies conjugated to Alexa Fluor 488 (A11034; Molecular Probes) or rhodamine (5 μg/mL; Southern Biotechnology) were used. Images were obtained with Bio-Rad Radiance 2000 laser scanning confocal or Nikon Eclipse E800 phase-contrast microscope.

Xenograft murine models. GFP-positive MM1S and Colo320 control shRNA and BCL9 shRNA cells (1×10^6) were injected into 6-week-old sublethally irradiated NOD.CB17-Prkdc^{SCID}/J mice (The Jackson Laboratory; i.v. for MM1S and i.p. for Colo320 cells). Animals were monitored daily and, when morbid, were euthanized; autopsies were done and tissues were formalin-fixed. GFP-positive tumors were visualized using the LT-9500 florescent light box (Lighttools Research). Experiments were approved by and conformed to the standards of the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute.

Histology, immunohistochemistry, and flow cytometry. Tissue sections were stained with H&E or incubated with 5 μg/mL primary antibodies or a corresponding IgG fraction of preimmune serum in 3% bovine serum albumin-PBS blocking solution for 16 h at 4°C. Immunohistochemical analysis were done using anti-BCL9 (ab37305; Abcam), anti-β-catenin (CAT5-H10; Zymed), anti-human CD44H (2C5; R&D Systems), and anti-mouse CD34 (RAM34; eBiosciences) and visualized with the corresponding biotinylated antibody coupled to streptavidin-peroxidase complex (Vector Labs). Flow cytometry was done as described previously (8).

ELISA. MM1S and Colo320 control shRNA and BCL9 shRNA cells (1×10^6) were cultured for 24 h in triplicates and VEGF levels in supernatants were measured by ELISA according to the manufacturer's instructions (DuoSet; R&D Systems). Standard curves were linear.

Results

Overexpression of BCL9 in multiple myeloma and colon carcinoma. *BCL9* mRNA expression levels were analyzed in several multiple myeloma and colon carcinoma primary tumors as well as in established cell lines. Expression analysis on Affymetrix gene chips revealed significantly higher *BCL9* transcripts levels in

[8] http://www.ncbi.nlm.nih.gov/geo/
[9] http://www.r-project.org
[10] http://bioconductor.org/packages/1.8/bioc/html/plier.html

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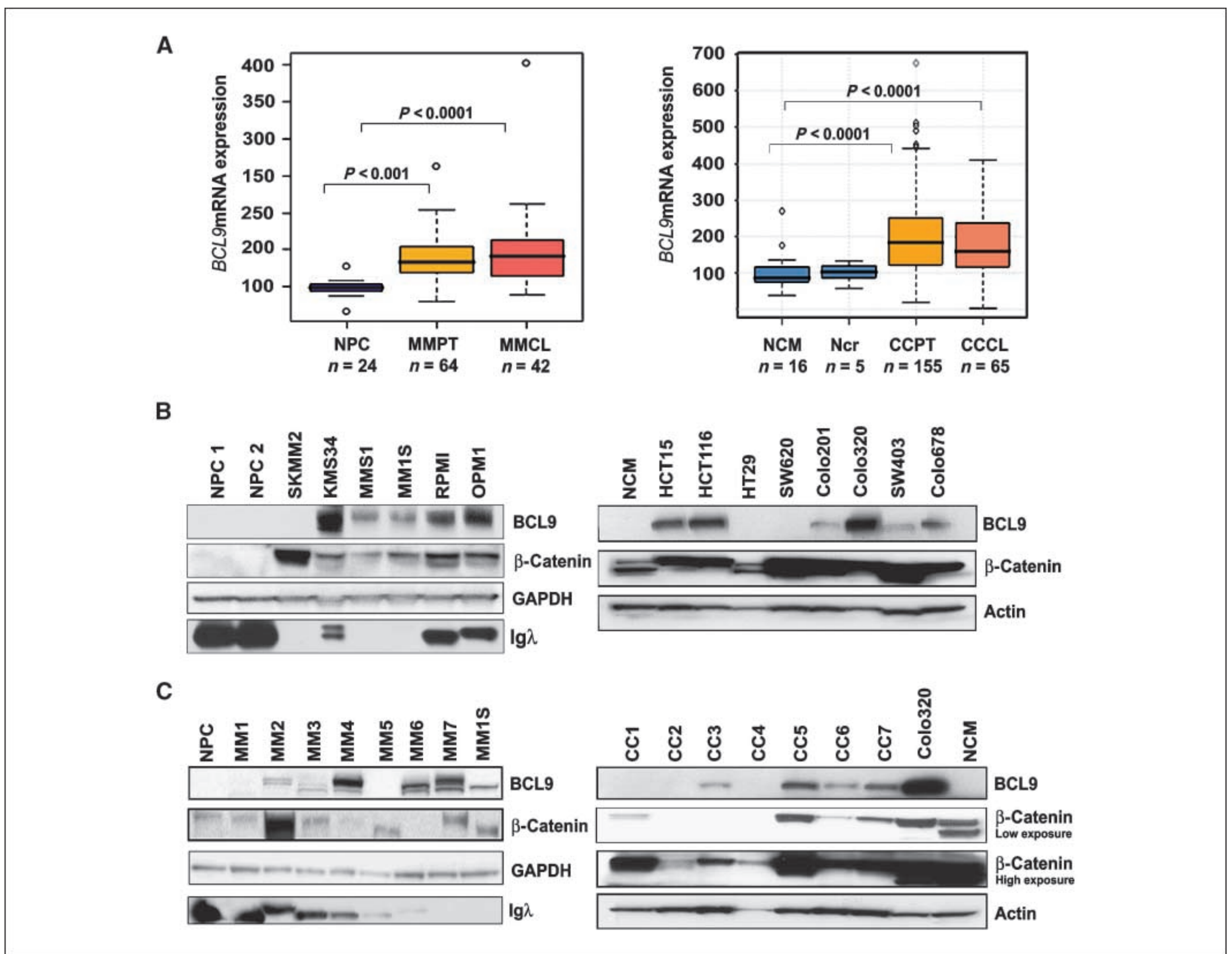

Figure 1. BCL9 is overexpressed in multiple myeloma and colon carcinoma. *A,* high *BCL9* mRNA expression in multiple myeloma primary tumors (*MMPT*) as well as multiple myeloma cell lines (*MMCL*) compared with normal plasma cells (*NPC*; *left*) and in colon carcinoma primary tumors (*CCPT*) as well as colon carcinoma cell lines (*CCCL*) compared with normal colonic mucosa (*NCM*) and normal crypt cells (*Ncr*; *right*). Immunoblot showing increased BCL9 protein expression in multiple myeloma (*left*) and colon carcinoma (*right*) cell lines (*B*) and primary tumors (*C*). Note that BCL9 protein expression in normal plasma cells and normal colonic mucosa is negligible. Igλ loading control is shown for normal plasma cells and multiple myeloma cells. Normal plasma cells secrete high levels of immunoglobulin, whereas multiple myeloma cells secrete varying immunoglobulin levels. MM1S (a multiple myeloma cell line) and Colo320 (a colon carcinoma cell line) were used as BCL9-expressing positive controls in the primary tumor Western blots.

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multiple myeloma primary tumors (60%, 38 of 64; $P < 0.001$) and multiple myeloma cell lines (64%, 27 of 42; $P < 0.0001$) compared with normal plasma cells ($n = 24$), the normal counterpart of multiple myeloma (Fig. 1*A, left*). *BCL9* mRNA levels were also significantly elevated in colon carcinoma primary tumors (68%, 106 of 155; $P < 0.0001$) and cell lines (65%, 42 of 65; $P < 0.0001$) compared with normal colon and normal colon crypt cells (Fig. 1*A, right*). The highest level of *BCL9* mRNA expression of normal plasma cells and normal colon cells was used as a stringent cutoff to determine the percentage of cell lines and primary tumors expressing elevated *BCL9* levels. High levels of *BCL9* mRNA were associated with increased levels of BCL9 protein in multiple myeloma (*left*) and colon carcinoma (*right*) cell lines as well as in primary tumors (Fig. 1*B* and *C*). Cell lines with elevated *BCL9* mRNA levels showed increased BCL9 protein expression (data not shown). Overall, these results document overexpression of *BCL9* in a subset of multiple myeloma and colon carcinoma.

BCL9 promotes proliferation, colony formation, migration, and the invasive potential of multiple myeloma and colon carcinoma cells. Transient or stable expression of BCL9 enhanced β-catenin–mediated transcription as well as cell proliferation in Colo320 (harbors adenomatous polyposis coli mutation), HCT15 (adenomatous polyposis coli mutation), HCT116 (β-catenin mutation), and myeloma cells (MM1S, OPM1, and MMS1), which have no known Wnt pathway gene mutations (Supplementary Fig. S1; data not shown; ref. 20). These results indicated that BCL9 overexpression may influence tumor cell proliferation and progression independent of the mutational status of the Wnt signaling components. To further investigate the involvement of BCL9 in different cellular functions, we used gain-of-function and loss-of-function approaches to either overexpress or knockdown BCL9 in several cell lines using lentiviral approaches (Fig. 2; Supplementary Figs. S1*C* and S2). To rule out that the observed BCL9 knockdown was not a consequence of off-target RNA

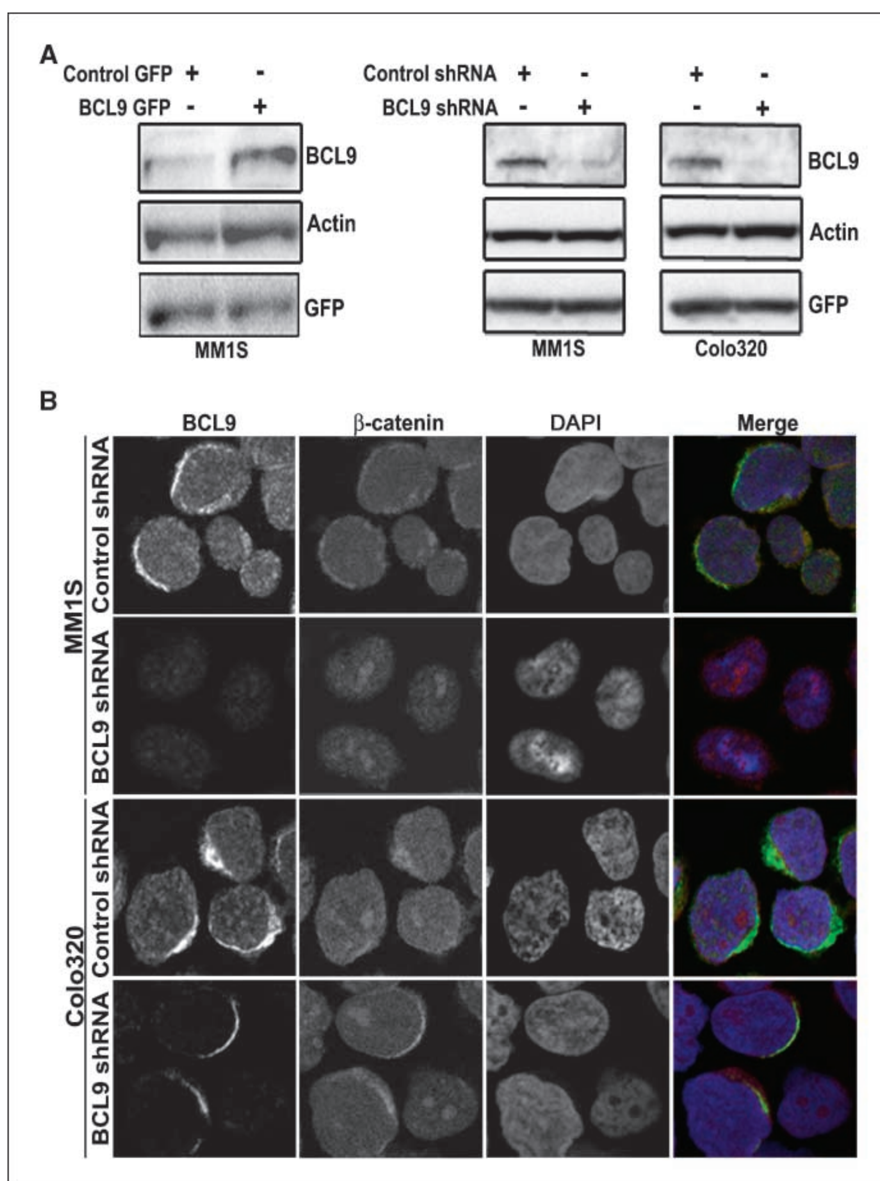

Figure 2. Gain-of-function and loss-of-function validation of BCL9 in tumor cell lines. *A,* BCL9 overexpression (*left*) with lentiviral BCL9 GFP and knockdown (*right*) with lentiviral BCL9 shRNA in multiple myeloma cell line MM1S and colon carcinoma cell line Colo320 as documented by immunoblot analysis. *B,* confocal immunofluorescence confirming knockdown of BCL9 expression in MM1S and Colo320 cells. Control shRNA and BCL9 shRNA expressing stable cells were sorted, fixed, and stained with indicated antibodies.

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interference effect, two distinct BCL9 shRNAs were tested in colon carcinoma as well as multiple myeloma cell lines and rescue experiments were done (Supplementary Figs. S2 and S3). The specificity of the anti-BCL9 polyclonal antibodies used in our studies were also rigorously validated (Supplementary Fig. S4).

We show that BCL9 knockdown reduced MM1S and Colo320 cell proliferation by 1.3- and 1.5-fold, respectively, compared with control shRNA (scrambled shRNA sequence) cells (Fig. 3*A*). In addition, overexpression of BCL9 in MM1S cells significantly increased the colony-forming ability, whereas BCL9 knockdown decreased the colony-forming activity of MM1S and Colo320 cells (Fig. 3*B*). The ability of BCL9 knockdown to reduce cell numbers could result from either decreased cell cycle progression or increased apoptosis. BCL9 knockdown decreased cell proliferation but did not significantly alter cell survival under multiple stress conditions (Fig. 3*A*; data not shown). The Wnt pathway has been implicated in cell migration of multiple myeloma cells and we therefore investigated whether MM1S cells with either overexpression or knockdown of BCL9 protein migrated differently in response to the Wnt3A ligand in a Transwell migration assay (10). BCL9 overexpression increased MM1S cell migration, whereas BCL9 knockdown significantly reduced migration (Fig. 3*C*). Additionally, a 5-fold decrease in adhesive and invasive potential of Colo320 BCL9 shRNA cells was observed in Matrigel-coated invasion chambers (Fig. 3*D*). Taken together, these results show that BCL9 is an important factor in determining the proliferation, migration, invasion, and the metastatic potential of multiple myeloma and colon carcinoma cells.

Role of BCL9 in tumor metastasis, survival, and EMT. The above *in vitro* results prompted us to investigate whether BCL9 affects survival in xenograft mouse models of multiple myeloma and colon carcinoma. Therefore, MM1S as well as Colo320 control shRNA and BCL9 shRNA cells were injected into sublethally irradiated NOD/SCID mice. BCL9 knockdown significantly increased the survival of mice injected with Colo320 BCL9 shRNA cells (Fig. 4*A*). The mean survival for mice with Colo320 control shRNA and BCL9 shRNA cells was 50 versus 71 days, respectively ($P < 0.0001$). Frequent intraparenchymal GFP-positive nodules as

well as tumor metastasis in the liver and kidney were observed in control shRNA engrafted animals (Fig. 4*B*). BCL9 shRNA engrafted animals showed small cellular aggregates and isolated tumor cells within the parenchyma, suggesting that knockdown of BCL9 efficiently attenuates tumor metastasis and tumor load. In the case of the multiple myeloma xenograft model, all animals transplanted with MM1S control shRNA cells developed hind limb paralysis and died by days 19 to 23 (Fig. 4*A*). Animals transplanted with MM1S BCL9 shRNA cells had a mean survival of 30 days compared with 21 days in control mice ($P = 0.0001$). The control shRNA myeloma cells metastasized to the bone marrow and showed massive tumor burden in head, spine, and long bones (Fig. 4*C*). Histologic examination of GFP-positive tissues in these control mice confirmed the presence of an increased tumor load in the bone marrow of spine that extended into the peridural space, accounting for the earlier onset of limb paralysis in control animals. Mice engrafted with MM1S BCL9 shRNA cells showed fewer GFP tumor nodules, decreased tumor load with smaller foci of cells infiltrating

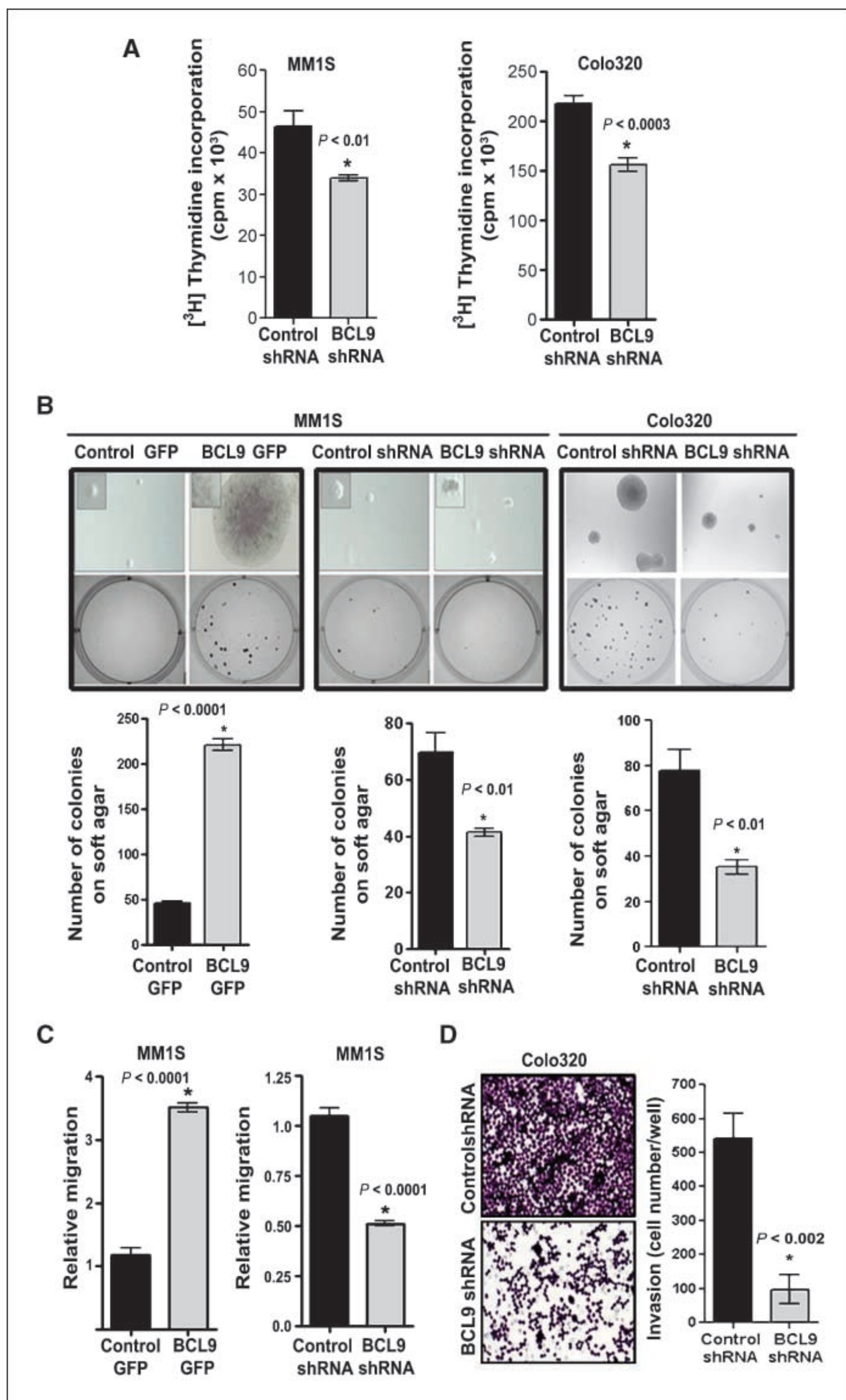

Figure 3. BCL9 enhances tumor cell proliferation, migration, and invasion. Effect of BCL9 knockdown on tumor cell proliferation (*A*), anchorage-independent soft-agar colony formation (*B*), cell migration (*C*), and cell invasion (*D*) in MM1S and Colo320 cells. MM1S cells stably overexpressing BCL9 GFP shows change in colony morphology and increase in cell migration compared with control GFP cells. *Bars,* SE.

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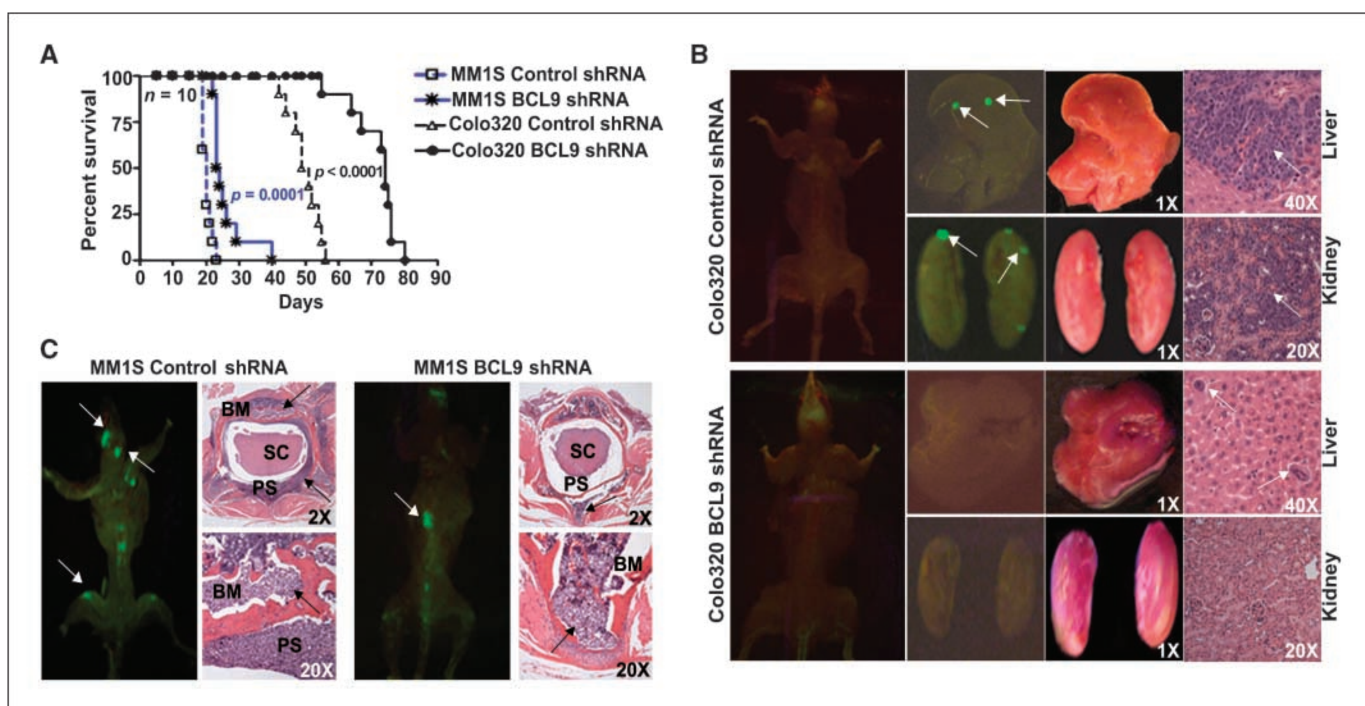

Figure 4. BCL9 knockdown increases survival and decreases tumor metastasis in xenograft mouse models of cancer. *A,* Kaplan-Meier survival curve of mice engrafted with MM1S (multiple myeloma cells) as well as Colo320 (colon carcinoma cells) control shRNA and BCL9 shRNA cells. Whole-body imaging and histologic analysis of engrafted mice with Colo320 (*B*) and MM1S (*C*) cells. Note decreased tumor burden in the liver and kidneys of mice with Colo320 BCL9 shRNA knockdown cells (*white arrows*) and peridural space (*PS*) of mice engrafted with MM1S BCL9 shRNA cells (*black arrows*). *SC,* spinal cord; *BM,* bone marrow.

the bone marrow, and preservation of the peridural space. Multiple myeloma is an aggressive blood cancer that spreads rapidly to the brain as well as spinal cord and may be less likely to depend on EMT for migration and metastasis compared with colon carcinoma cells, which may explain the difference in the survival between these two xenograft models. The importance of EMT in metastasis of epithelial tumors led us to investigate the involvement of BCL9 in EMT. Knockdown of BCL9 altered the subcellular distribution of epithelial and mesenchymal markers, β-catenin, E-cadherin, and vimentin (Supplementary Fig. S5*A* and *B*). Overall, these results show the involvement of BCL9 in EMT, tumor metastasis, and survival.

BCL9 target genes CD44 and VEGF mediate metastasis and angiogenesis during tumor progression. To further delineate the role of BCL9 in tumor invasion and metastasis and to gain insight into the possible Wnt target genes regulated by BCL9, we performed expression profiling analysis. Principal Component Analysis of Affymetrix chips revealed good replicates of the samples, with clear grouping by cell type (multiple myeloma versus colon carcinoma) as well as treatment (control shRNA versus BCL9 shRNA; Supplementary Fig. S6*A*). Using Limma moderated t test with adjusted $P < 0.05$, we detected 76 known genes clustered into four different groups that were differentially expressed in BCL9 shRNA compared with control shRNA cells (Fig. 5*A*; refs. 15, 16). Interestingly, among the 28 down-regulated genes were two known Wnt target genes, VEGF and CD44 (21, 22). BCL9 knockdown reduced *c-Myc, cyclin D1, CD44*, and *VEGF* mRNA expression levels in multiple myeloma and colon carcinoma cells (Fig. 5*B*). A decrease in c-Myc and cyclin D1 protein expression was also observed on down-regulation of BCL9 (Fig. 5*C*). To further validate the expression profiling and quantitative reverse transcription-PCR results, we characterized the expression of CD44 and VEGF by immunohistochemical analysis and ELISA, respectively. Tissue sections from colon carcinoma mouse xenografts showed strong CD44 staining in the frequent metastatic tumors that developed in animals transplanted with control shRNA cells and very weak staining in the tumor cells of the few metastatic nodules detected in animals transplanted with BCL9 shRNA cells (Fig. 5*D*). Down-regulation of BCL9 expression reduced VEGF secretion by tumor cells as well as *in vitro* capillary tube–forming ability of multiple myeloma and colon carcinoma cells (Fig. 5*E*; Supplementary Fig. S6*B*; ref. 23). Immunohistochemical analysis using an anti-mouse CD34 antibody that recognizes endothelial cells of murine blood vessels showed a significant reduction in the number of blood vessels formed within the tumor cells and decrease in the intensity of CD34 staining in the BCL9 shRNA multiple myeloma xenograft mice (Fig. 5*F*). These findings indicate that BCL9 promotes tumor metastasis and its effects on angiogenesis are mediated through CD44 and VEGF.

Aberrant BCL9 expression is associated with tumor progression in multiple myeloma and colon carcinoma. The above results prompted us to investigate the pattern of BCL9 expression in a panel of normal human tissues and tumors to understand its role in tumor progression. Immunohistochemical studies on normal plasma cells (n = 10), normal colonic mucosa (n = 10), multiple myeloma bone marrow biopsies (n = 30), and colon tumor samples (n = 10) showed increased nuclear BCL9 levels in one third of multiple myeloma and colon carcinoma samples compared with their normal cellular counterparts (Fig. 6). BCL9 expression was enhanced in the nucleus of multiple myeloma cells, whereas β-catenin expression was localized to perinuclear region in these samples; in contrast, normal plasma cells showed minimal BCL9 and β-catenin staining (Fig. 6*A*). In agreement with our RNA expression profiling studies, BCL9 protein expression

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was predominantly nuclear in colon carcinoma, whereas the normal crypt cells were negative for BCL9 staining (Fig. 6*B*). However, some of the stromal cells in normal colon stained positive for BCL9 (Fig. 6*B*), indicating that it may play a crucial role in tumor stromal interactions and provide proliferative signals. Highest levels of BCL9 expression were detected at the invasive front edge of the tumors where dislodging of micrometastases and blood vessel penetration take place (Fig. 6*C*; Supplementary Fig. S7*A*; data not shown), indicating a role for BCL9 in tumor invasion and metastasis. Increased nuclear BCL9 expression was also detected in four cases of melanoma at vertical growth phase (Supplementary Fig. S7*B*). Nuclear colocalization of β-catenin was also detected at the front edge of the invading tumors (Fig. 6*C*). In the premalignant colonic adenomatous polyps analyzed ($n = 6$), nuclear BCL9 and nuclear β-catenin expression were observed in all cases (Fig. 6*D*). On the other hand, the stalk of the polyp, which contains normal colonic mucosa, showed no BCL9 staining, whereas β-catenin staining was predominantly cytoplasmic and in the membrane. The expression of BCL9 in the colonic adenomatous polyps and in areas of micrometastasis in tumors

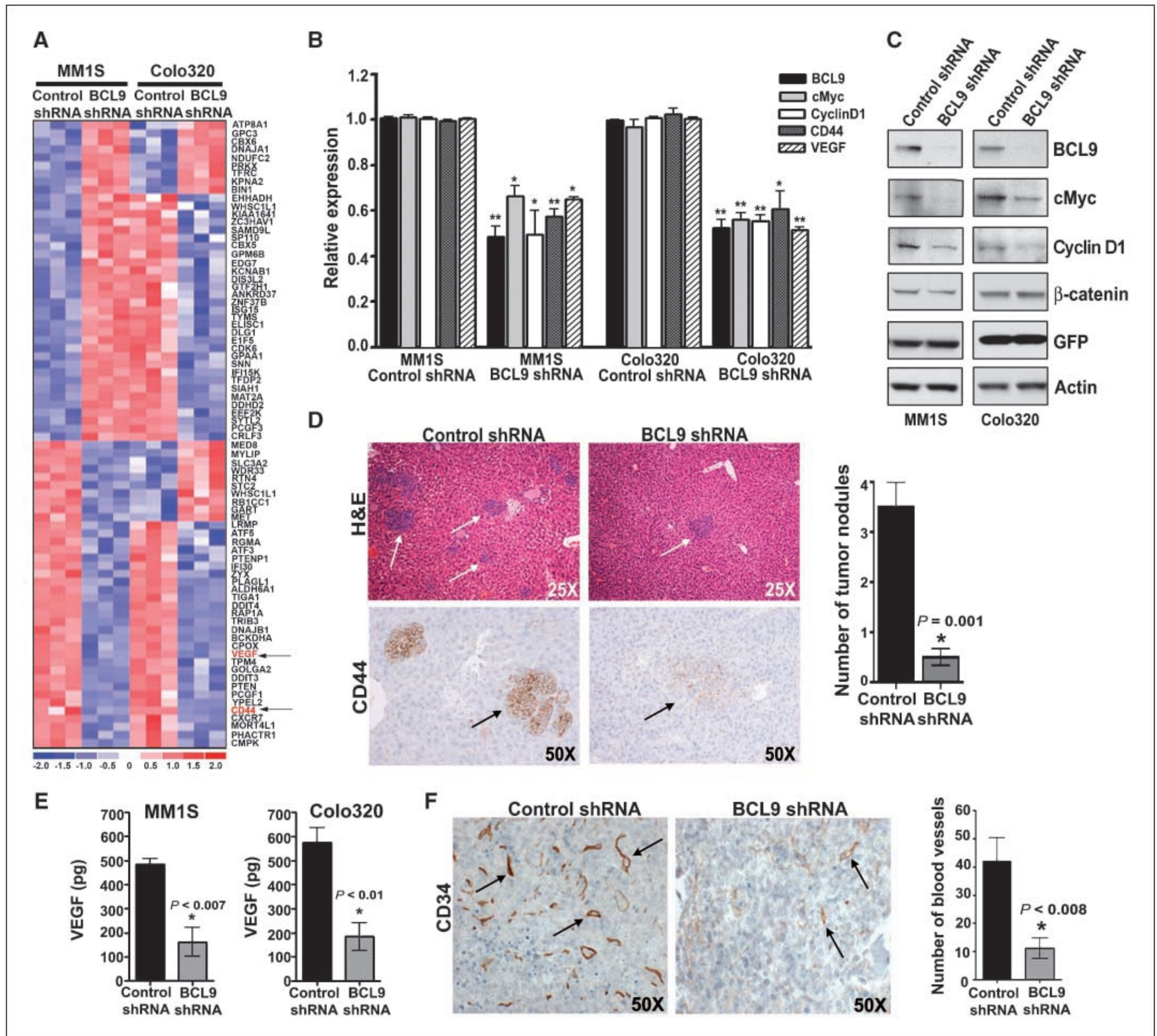

Figure 5. BCL9 is involved in tumor metastasis and angiogenesis. *A,* heat map of expression profiles of MM1S and Colo320 stably expressing control shRNA or BCL9 shRNA. Genes altered on knockdown of BCL9 are indicated. Down-regulation of CD44 and VEGF, known Wnt target genes, are indicated in red and by arrows. *B, BCL9, c-Myc, cyclin D1, CD44*, and *VEGF* expression in MM1S and Colo320 cells following BCL9 shRNA knockdown as measured by quantitative reverse transcription-PCR. *, $P < 0.01$; **, $P < 0.0001$. Nontargeting control shRNA was used as control. *C,* down-regulation of BCL9 by lentiviral BCL9 shRNA decreases c-Myc and cyclin D1 but not β-catenin protein expression in MM1S and Colo320 cells. *D,* decreased metastasis, tumor nodules (*white arrows*), and CD44 (*black arrows*) expression in mice engrafted with Colo320 BCL9 knockdown cells compared with control. *E,* BCL9 knockdown decreases VEGF secretion in MM1S and Colo320 cells as measured by ELISA. *F,* down-regulation of BCL9 decreases host angiogenesis in mice injected with MM1S BCL9 shRNA cells as measured by decreased mouse CD34 staining (*black arrows*). Mouse anti-CD34 stains the endothelial blood vessel cells. The number of blood vessels formed in mice injected with MM1S BCL9 shRNA is significantly decreased.

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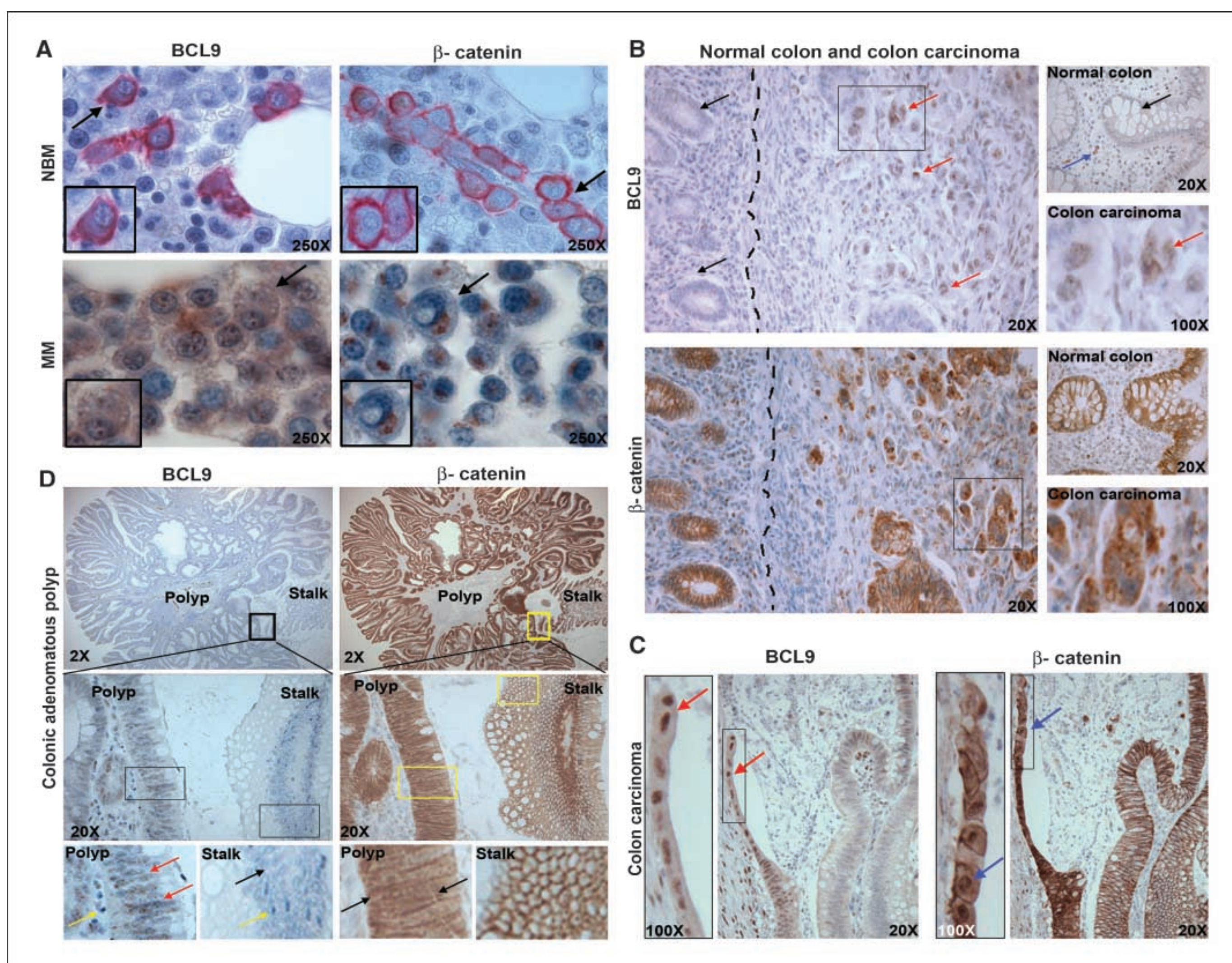

Figure 6. Immunohistochemical analysis of BCL9 expression in multiple myeloma and colon carcinoma primary tumors. *A,* increased nuclear localization of BCL9 in multiple myeloma (*MM*) compared with normal bone marrow (*NBM*) plasma cells. Normal bone marrow cells are costained with anti-CD138 (*red*) and anti-BCL9 (*brown*) or anti-β-catenin (*brown*). *Arrows,* cells in the insets. *B,* nuclear BCL9 expression in colon carcinoma. The dashed black line demarcates normal colon (*black arrows*) and tumor (*red arrows*). Note that normal colon crypt cells are negative for BCL9 (*top inset, black arrow*), whereas some stromal cells are positive (*blue arrow*). Nuclear β-catenin expression in adjacent tissue sections is also shown (*bottom*). Areas in the black boxes are enlarged (*right, bottom inset*). *C,* up-regulation of nuclear BCL9 (*red arrows*) and β-catenin (*blue arrows*) in the invasive regions of colon carcinoma. *D,* increased nuclear BCL9 (*brown staining* and *red arrows*) and β-catenin staining (*brown staining* and *black arrows*) in colonic adenoma polyps. Areas in the black and yellow boxes are enlarged below. Note the absence of BCL9 expression and conspicuous cytoplasmic and membrane β-catenin expression in the stalk (*bottom inset*). The blue staining seen in the stalk and some stromal cells (*yellow arrows*) corresponds to counterstaining with hematoxylin.

as well as stromal cells suggests a role for BCL9 in progression at different tumor stages that may be mediated through autocrine or paracrine mechanisms. Collectively, our findings indicate that BCL9 is involved in the progression of a broad spectrum of cancers.

Discussion

Aberrant activation of the Wnt pathway due to gene mutations, retention of β-catenin in the nuclei, epigenetic factors that silence the secreted Frizzled-related protein and Wnt inhibitory factor, and overexpression of Wnt ligands and Frizzled receptor genes have been reported in several common human carcinomas (6, 24–26). Constitutively active canonical Wnt/β-catenin pathway has been documented in multiple myeloma and other hematologic malignancies, although no mutations have been identified, suggesting that mechanisms other than gene mutations may contribute to Wnt pathway deregulation. Here, we provide evidence that, in multiple myeloma and colon carcinoma, dysregulation of BCL9 expression is a novel oncogenic mechanism of Wnt pathway activation.

Several genes located on chromosome 1q, including *BCL9, CKS1B, PDZK1, MUC1*, and *RAB25*, are frequent targets of amplification and are often associated with increased gene dosage and aberrant expression (12, 13, 27–30). Our study shows that BCL9 expression is negligible in normal plasma and normal colon mucosa cells, whereas it is highly expressed in a subset of multiple myeloma and colon carcinoma cells. The marked *BCL9* overexpression may be due to an increase in gene dosage of the *BCL9* gene. However, when we analyzed *BCL9* DNA copy number and mRNA expression in multiple myeloma patients, although

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a positive relationship was observed (data not shown), this correlation was low, indicating that additional mechanisms other than increased gene dosage may be involved in BCL9 up-regulation. Previously, it has been shown that the removal of an inhibitory effect imposed by chromosome 1q12 heterochromatin when *BCL9* is translocated to another region of the genome (positional effect) could also result in aberrant BCL9 expression (31, 32). In another study, it was shown that BCL9 is a Wnt-inducible β-catenin target gene and this positive feedback loop may account for its up-regulation in tumor cells (16). Further studies are needed to determine whether *BCL9* gene mutations, methylation, or post-translational modifications or all the above may contribute to additional modes of BCL9 deregulation.

There is increasing evidence for the role of critical pathways involved in the regulation of metastasis and how tumor cells can conscript these pathways to create a microenvironment that enhances their ability to metastasize. Our studies in the xenograft mouse models of cancers showed a dramatic influence of BCL9 in mouse survival as well as tumor cell metastasis. We provide evidence for the involvement of BCL9 in promoting EMT, a phenomenon involved in the metastasis and progression of tumors. Our expression profiling data on BCL9 knockdown cells revealed down-regulation of a subset of genes involved in EMT, including *PTEN, Zyxin*, and *RAP1A* (Fig. 5*A*; refs. 33, 34). Furthermore, the expression of vimentin, a mesenchymal marker, E-cadherin, as well as β-catenin epithelial markers were modulated by BCL9, whereas another EMT modulator, snail, was not, supporting our suggestion that BCL9 may be involved in the regulation of a subset of EMT regulators (35). Based on our observations, we propose a model wherein aberrant deregulation of BCL9 confers mesenchymal characteristics to tumor cells, which enhances cell migration, invasion, angiogenesis, and metastasis (Supplementary Fig. S5*C*). Indeed, we show that BCL9 is highly expressed in the advancing edge and invasive regions of colon carcinoma and in the vertical growth phase of malignant melanomas that are associated with increased invasive and metastatic potential (Fig. 6*C*; Supplementary Fig. S7; ref. 36). Our results are in agreement with previous studies documenting a role of B9L, a relative of human BCL9, in promoting EMT of nontransformed MDCK cells (37). Up-regulation of human B9L expression has been reported in colon and breast cancers and has been implicated in tumorigenesis induced by aberrant activation of Wnt signaling (38–40). Consistent with these finding, we observed B9L expression in colon carcinoma as well as multiple myeloma cell lines (Supplementary Fig. S8); however, the B9L expression pattern is distinct compared with BCL9 expression in these cells (see Fig. 1*B*). *BCL9* and *B9L* genes reside on chromosome 1q21 and 11q23.3, respectively. These regions are targets of recurrent chromosomal alterations in multiple myeloma and colon carcinoma, suggesting that the molecular mechanisms underlying BCL9 and B9L regulation may be very different.

Our gene expression profiling studies identified two known Wnt target genes, VEGF and CD44, that are down-regulated by knockdown of BCL9. BCL9 has recently been shown to possess a COOH-terminal transcriptional activation domain that may function in a cell type–specific manner (15, 16). This may explain the results of differentially regulated target gene expression in multiple myeloma and colon carcinoma BCL9 knockdown cells. We have established that BCL9 plays an important role in tumor progression by regulating CD44-mediated tumor metastasis as well as VEGF-mediated host angiogenesis. However, it remains to be identified whether this regulation is influenced by BCL9 COOH-terminal ligand(s). Interestingly, CD44 expression is up-regulated in several tumors and has been associated with chromosome 1q amplification as well as tumor progression in breast carcinoma (41). Given these data and the genomic location of *BCL9*, one could speculate that BCL9 may be amplified and overexpressed in these breast tumors, leading to up-regulation of CD44 and tumor progression of breast cancer and other malignancies associated with chromosome 1q amplification. There is also mounting evidence that VEGF plays a pivotal role in the proliferation of endothelial cells in the vascular-rich tumor microenvironment, promoting tumorigenesis via angiogenesis. Interestingly, the VEGF promoter has recently been shown to consist of two TCF binding sites, lending support to our findings on BCL9 regulation of VEGF (42). Attenuation of VEGF secretion by tumor cells and suppression of tumor vasculature formation on knockdown of BCL9 as documented in our studies highlights the need for the development of BCL9 inhibitors to arrest tumor angiogenesis and progression.

Small-molecule inhibitors and antibodies targeting crucial Wnt protein complexes, downstream target genes, as well as activators and ligands of the Wnt pathway have been proposed as potential tools for therapeutic intervention against cancer cells (6). A major potential limitation of therapies that aim to target Wnt/β-catenin is its importance in normal adult tissue functions such as stem cell regeneration, wound healing, and cell proliferation (43). Our findings that BCL9 expression is negligible in normal plasma cells and normal colon cells compared with multiple myeloma and colon carcinoma cells may provide a rationale for targeting BCL9 in cancers without majorly affecting the normal stem cell compartment. This possibility is highlighted by the recent structural elucidation of the interactions between BCL9/β-catenin/TCF4, suggesting that disruption of the BCL9/β-catenin interaction carries the potential for selective therapeutic interventions (44).

In summary, this study provides critical insight into the role of the BCL9 oncogene in the progression of multiple myeloma and colon carcinoma. We have shown that BCL9 plays fundamental roles in tumor progression by regulating proliferation, migration, invasion, angiogenesis, and the metastatic potential of tumor cells. The pleiotropic roles of BCL9 and its up-regulation in a variety of frequent human cancers highlight its importance as a novel therapeutic target in the treatment of cancers associated with deregulated Wnt signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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