Bcl-2 is a strongly antiapoptotic protein that is expressed in normal melanocytes, benign nevi, primary melanoma, and melanoma metastases (1–3). Because of the ability of this protein to decrease the apoptotic response to cytotoxic chemotherapy (4–8), Bcl-2 has been a target for small molecule and antisense oligonucleotide development (9). G3139, an 18-mer phosphorothioate oligodeoxynucleotide targeted to the initiation codon region of the Bcl-2 mRNA, has been shown to down-regulate Bcl-2 protein and mRNA expression in melanoma and other cells in vitro (10–13). This drug has completed worldwide phase III clinical trials in combination with dacarbazine (DTIC) alone (Genta). However, questions have arisen over the mechanism of action of this agent both in the in vitro (12, 13) and in vivo (15) settings, and indeed, of the role of Bcl-2 protein in preventing apoptosis in melanoma cells, which, unlike follicular lymphoma cells, do not have a t14:18 translocation.
protein and mRNA were down-regulated both in the cytoplasm and mitochondria by >90%, but chemosensitization to a variety of cytotoxic agents was not observed (12). Indeed, no cellular phenotypic changes could be identified. In striking contrast, treatment of 518A2 melanoma cells in vitro with G3139 induces profound cellular apoptosis that has been shown to be entirely Bcl-2 independent (13). Our recent data suggest that the apoptosis in G3139-treated melanoma cells may be due to a direct interaction between G3139 and a protein in the outer mitochondrial membrane known as voltage-dependent anion selective channel (VDAC, ref. 16). This interaction, which does not occur between VDAC and the anti-Bcl-2 siRNA, causes a change in conformation of VDAC, which leads to loss of conductance of small anions, such as ADP, through it. The binding of VDAC to G3139 also leads to the opening of another channel on the outer mitochondrial membrane, through which cytochrome c, an initiating factor in the apoptotic process (13), can be released (17).

Therefore, because siRNA-induced Bcl-2 silencing produces no phenotypic changes and no chemosensitization in 518A2 melanoma cells and the proapoptotic effects of the antisense anti-Bcl-2 oligomer G3139 are independent of Bcl-2 expression, no clear antiapoptotic function, nor indeed any anti-Bcl-2 oligomer G3139 are independent of Bcl-2 expression (13, 16), this interaction, which does not occur between VDAC and the anti-Bcl-2 siRNA, causes a change in conformation of VDAC, which leads to loss of conductance of small anions, such as ADP, through it. The binding of VDAC to G3139 also leads to the opening of another channel on the outer mitochondrial membrane, through which cytochrome c, an initiating factor in the apoptotic process (13), can be released (17).

Therefore, because siRNA-induced Bcl-2 silencing produces no phenotypic changes and no chemosensitization in 518A2 melanoma cells and the proapoptotic effects of the antisense anti-Bcl-2 oligomer G3139 are independent of Bcl-2 expression, no clear antiapoptotic function, nor indeed any function, for Bcl-2 protein could be identified in these cells in vitro. On the other hand, in the in vivo setting, evidence exists that G3139 causes a significant diminution in tumor volume when used to treat immunocompromised mice bearing 518A2 melanoma xenografts (10, 18). These data suggest that the function of Bcl-2 with respect to 518A2 melanoma cell viability in the in vivo setting may be significantly different than in the in vitro setting. To reconcile these differences, we constructed a plasmid (pssXF-BCL2-AS) that, when transfected, constitutively expresses a short single-stranded antisense oligodeoxyribonucleotide complementary to the initiation codon region of the Bcl-2 mRNA (the control is the sense plasmid pssXF-BCL2-S). This provides for the stable long-term silencing of Bcl-2 protein and mRNA expression. We also knocked down the expression of Bcl-2 protein in 518A2 melanoma cells by means of a stably expressed short hairpin RNA (shRNA). Our data show that both techniques used to knock down Bcl-2 protein expression produce clones that behave almost identically to the control clones in vitro, but that their ability to grow tumors in severe combined immunodeficient mice bearing 518A2 melanoma xenografts dramatically differs from that of the controls.

Materials and Methods

Cells
The 518A2 Mycoplasma-free human melanoma cell line was a kind gift of Dr. Volker Wachek (University of Vienna, Vienna, Austria). The cells were grown in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate. Stock culture was maintained at 37 °C in a humidified 5% CO2 incubator.

Reagents
Fetal bovine serum and Lipofectamine 2000 were purchased from Invitrogen. The anti-Bcl-2 monoclonal antibody was from Dako (Carpinteria, CA). The anti-α-tubulin monoclonal antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich (St. Louis, MO). The anti–poly(ADP-ribose) polymerase, anti–caspase-3 monoclonal antibody, anti-Bax, and anti-Bcl-xl were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti–cytochrome c monoclonal antibody and anti-Bak were from Upstate (Lake Placid, NY). The anti-Akt and anti–phospho-Akt antibodies were from Cell Signaling Technology (Danvers, MA). Phosphorothioate oligonucleotides (G3139 and two-base mismatch control for G3139) were synthesized and purified via standard procedures and kindly supplied by Genta (Berkeley Hills, NJ).

Vector constructions
pssXF vector producing antisense ssDNA targeted to the Bcl-2 mRNA. This vector was constructed following the method of Chen and McMicken (19). To create a multiple cloning site, two oligodeoxyribonucleotides, 5′-E/S/P-P-linker (5′-TGAGACGCGCCAGGTGCTTCGCGATCCGGAGCCGACGCCCGCATGCCGGTGTTTCGTCCTTTCCACAAATAGATCCCGAGGCCTGTCAGTTAAT-3′) and 3′-E/S/P-P-linker (5′-TAAC-GGCGGCGAAGCCGTTTCCGGCGCGCTG3′), were annealed and inserted into the PacI and XhoI sites of a ssDNA expression vector, pssXD (19). This new construct was designated as pssXF. To generate the anti-Bcl-2 oligodeoxyribonucleotide, called BCL2A, in cells, two oligodeoxyribonucleotides, 5′BCL2A(E/P) (5′-ATATTITCCCGGTGTTGTGCACCTTTCTCCGTGGCGTCCCTAT-3′) and 3′BCL2A(E/P) (5′-ATGCGCCGCCTGGGAGACCGGTAGCAGACCGCGGA-3′), were annealed and subcloned into the EcoRI and PacI sites of the pssXF vector. The antisense oligodeoxyribonucleotide was designed to target the 5′ ATG region of the Bcl-2 mRNA. The resulting construct was designated pssXF-BCL2A-AS. The control vector pssXF-BCL2-S was similarly constructed. The sequences of the inserted oligodeoxyribonucleotides were 5′BCL2S(E/P) (5′-AATATCGGACCACCGGTAGCAGACCGCGGA(A)-3′), 3′BCL2S(E/P) (5′-ATCGCCGCCTGGGAGACCGGTAGCAGACCGCGGA-3′), and 5′-TCTCCCGCGTCTGGATCCTCTGCACCGCGGATG3′.

The pssXF plasmid thus contains a Moloney mouse leukemia viral reverse transcriptase primer binding site, the Moloney murine leukemia virus reverse transcriptase gene, the target sequence, and a stem-loop structure. All of these elements are transcribed into a single mRNA molecule. Newly synthesized reverse transcriptase, after binding of endogenous tRNA18S to the 3′ terminus of the transcript, reverse transcribes the ssDNA target sequence (i.e., Bcl-2), until transcription is terminated by the stem-loop structure. Subsequently, the ssDNA is released after template degradation either by endogenous RNase H or by the RNase H activity of the reverse transcriptase (19, 20).

dDNA vector encoding shRNA targeted to the Bcl-2 mRNA. The U6-driven casette expressing the Bcl-2 shRNA was obtained using a PCR reaction as previously described (21). Briefly, the 5′ PCR primer is complementary to 25 nucleotides at the 5′ end of the U6 promoter and it includes a BamHI restriction site added to its 5′ end for cloning purposes. The 3′ end of the 3′ PCR primer is complementary to 30 nucleotides at the 3′ end of the U6 promoter, followed by the sense strand, a TTGTTGTAG loop, the antisense, and the terminator sequence (8 units). A plasmid containing the U6 promoter was used as template in the PCR reactions and amplified under the following conditions: 1 minute at 94 °C, a 4-minute ramp down to 60 °C, 1 minute at 60 °C, and 1 minute at 82 °C, for 30 cycles using Vent Polymerase. The resulting PCR products were purified with QiaQuick columns (Qiagen, Valencia, CA) and was cloned into the pcDNA3.1 vector to produce pcDNA3.1BCL2A. The empty vector was used as a control. The sequences of the oligonucleotides employed in the PCR reactions are as follows: 5′-CTAGCTGATCCAAAGGTCGCTGGCAGAAGGGCCCTAT-3′, 3′ PCR primer, 5′-CTATGTTGGGAAACATGGCGCTTCTTTGATCACTACA-CAATATCAACAGGGCGATCCGGGTGTTCCTGCTCTTTCCACACAGTATATAAAGCC-3′.
For the shRNA design, the original siRNA target sequence in the Bcl-2 transcript was extended one nucleotide at its 5' end. The resulting double-stranded region in the shRNA is 20 nucleotides in length; however, the first two nucleotides of the shRNA loop sequence can potentially form basepairs. Thus, it is possible that during intracellular processing of the shRNA molecule, one or two additional nucleotides from the loop sequence would be added to the shRNA antisense sequence. These additional nucleotides have the ability to pair with the target site sequence, further extending it at its 5' end (target, 5'-AGC-GATGGCCGCCTCTGGTAGATT-3'; predicted antisense sequence, 5'-AGT-CAAACAGAGGCCGCATGC-3').

**Plasmid transfection**

Cells were seeded the day before the experiment in six-well plates at a density of 13 x 10^4 to 15 x 10^4 per well to be 60% to 70% confluent on the day of the transfection. All transfections were done in Opti-MEM medium (Invitrogen) plus complete medium without antibiotics as per instructions of the manufacturer. The appropriate quantities of reagents were diluted in 100 µL of Opti-MEM medium to give a final concentration of Lipofectamine 2000 and plasmid. The solutions were mixed gently and preincubated at room temperature for 30 minutes to allow complexes to form. Then, 800 µL of complete medium without antibiotics were added and the solution was mixed and overlaid on the cells. The incubation time for oligonucleotide/Lipofectamine 2000 complexes was 5 hours, followed by incubation in complete medium containing 10% fetal bovine serum for 24 hours. The selection was achieved in the respective growth media supplemented with 1 mg/mL of geneticin (G418). Cells were then cultured for several weeks until genetin-resistant cells were developed as small colonies. Colonies were then selected and grown separately in fresh media supplemented with the selection antibiotic. Fifty to fifty-five colonies were screened and Bcl-2 expression was evaluated by Western blotting. The B10 (pcDNA3.1-shRNA) clone was selected for further study. The control was the empty vector clone (M10). For the pssXF-BCL2-AS–transfected cells, 90 colonies were screened and two clones, as32 and as34, were selected for further study.

**Western blot analysis**

Cells were harvested, washed in PBS, and then extracted in lysis buffer as previously described (22). Protein concentrations were determined with the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Aliquots of cell extracts, containing 15 to 100 µg of protein, were resolved by SDS-PAGE, transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL), and then probed with the appropriate antibodies. Enhanced chemiluminescence (Amersham) was done according to the instructions of the manufacturer.

**Isolation of RNA and reverse transcription-PCR**

Total RNA was isolated from 518A2 cells with Qiagen RNeasy kit, quantitated by UV absorbance. RNA was reverse transcribed with reverse transcription-PCR (RT-PCR) reactions based on the SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). All RT-PCR reactions were done as previously described (23). Bcl-2 was amplified with 1 µg of total RNA. The forward primer was 5’-GGTGGCACCCTCTGGTCGACCTAGT-3’ and the reverse primer was 5’-CTCTACCTGTTGCCCCAGAATA-3’. The Bcl-2 amplicon was 459 bp.

The primers (forward, 5’-GACGCTGGCTGCTGGGGGAGG-3’; reverse, 5’-CCGAGATGGCAGGGGAGGCTACCC-3’) designed to amplify a 246-bp fragment of β-actin were used to normalize for RNA concentration. The RT-PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

**Mitochondria isolation and treatment with G3139**

The pssXF-BCL2-AS and pssXF-BCL2-S clones were harvested by trypsinization and were washed with cold PBS. Cell pellets were resuspended in 300 mL of buffer A [250 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EGTA, 50 mg/mL Pefabloc, and 15 mg/mL leupeptin, aprotinin, and pepstatin]. Cells were then homogenized on ice in a dounce homogenizer until ~90% of cells were disrupted, as judged by trypan blue staining. Crude lysates were centrifuged twice at 1,000 x g for 10 minutes at 4°C to remove nuclei and unbroken cells. The supernatant was collected in a new tube and was subjected to centrifugation at 10,000 x g for 30 minutes at 4°C. The mitochondrial pellets were treated with increasing concentrations of G3139 in 20 µL of buffer B for 2 hours at 10°C. The supernatants were then mixed with loading buffer and Western blot analysis was done as described above.

**Growth of human tumor xenografts in severe combined immunodeficient mice**

Male severe combined immunodeficient ICR mice, 4 to 6 weeks old, were obtained from Taconic Farms (Germantown, NY). Animals were housed in barrier microisolator cages in laminar flow racks and received autoclaved food and water. All studies were conducted under a protocol approved by the Albert Einstein College of Medicine Institutional Review Board. Mice were injected s.c. into the right lower flank with 5 x 10^6 stably transfected cultured human 518A2 melanoma cells per mouse [pssXF-BCL2-AS (6 mice); pssXF-BCL2-S (5 mice); B10 and M10 (10 mice)] resuspended in 100-µL PBS plus 100-µL Matrigel (BD Biosciences, Bedford, MA). After tumors became visible, tumor size was measured every 2 to 3 days. Caliper measurements of tumor sizes were converted into mean tumor volume (mm^3) using the formula V = (A x B^2)/6, where A is the length of the largest dimension of the tumor and B is the length of the tumor perpendicular to A. The Mann-Whitney test was used to determine the statistical significance between two groups of animals. Data were considered significant at P < 0.05.

For Western blotting analysis of Bcl-2 protein expression in human melanomas xenografted in mice, the frozen tumor samples were homogenized in liquid nitrogen and processed as described above for cultured cells.

**Results**

**Bcl-2 protein and mRNA down-regulation**

**ShRNA targeted to the Bcl-2 mRNA.** The shRNA targeted against the Bcl-2 transcript was made with a one-step PCR strategy as previously described (ref. 24; Fig. 1A). The design of the shRNA was chosen to create minimal changes to the original 19-nucleotide target sequence in the Bcl-2 transcript originally targeted with synthetic siRNAs. We elected to extend the target site (5’-CATGCGGCGTCTGTTGGA-3’) one nucleotide at its 5’ end to facilitate the processing of the double-stranded looped shRNA. Thus, a guanine was added to the 5’ end of the Bcl-2 shRNA sense strand (5’-GGCATGCGGCGTCTGTTGGA-3’) because it is necessary to preserve a G as the +1 nucleotide to maintain the transcription strength of the U6 promoter. This additional G will basepair with the first U left by the termination signal, which in turn has the ability to pair with the A that precedes the original target site sequence in the Bcl-2 transcript. In cells transfected with the B10 (pcDNA3.1-shRNA) plasmid, multiple clones were isolated with >80% Bcl-2 down-regulation relative to the empty vector (M10) clone, as shown by Western blotting (Fig. 2A).

**Antisense DNA targeted to the Bcl-2 mRNA.** After transfection of the Bcl-2 plasmid, pssXF-BCL2-AS or pssXF-BCL2-S (Fig. 1B), into 518A2 melanoma cells and clonal selection, we determined the extent of Bcl-2 protein expression silencing by Western blotting. As is shown in Fig. 2B, several clones were
identified in which Bcl-2 protein expression was significantly reduced as measured by laser scanning densitometry (95% in two clones, as32 and as44; not shown) compared with expression in the pssXF-BCL2-S transfected 518A2 cells. Levels of many other apoptosis-related proteins, including Bak, Bax, Bcl-xL, poly(ADP-ribose) polymerase, Akt, and phospho-Akt, were unchanged (Fig. 2C). However, because down-regulation of Bcl-2 protein expression might have been...
an artifact of the pssXF plasmid transfection, we also evaluated Bcl-2 mRNA expression, which we expected to be diminished if the vector behaved as an antisense effector system. In fact, compared with the control sense clones, Bcl-2 mRNA expression was significantly reduced in the pssXF-BCL2-AS–transfected clones as well (Fig. 2D). Those two clones were chosen for further studies. In all cases, the expression of β-tubulin protein was unchanged. The down-regulation of protein expression in the pssXF-transfected clones was shown by Western blotting to be stable for at least 8 months, and for at least 6 months in the pcDNA3.1-shRNA or empty vector clones also grew at similar rates in tissue culture. However, the 518A2 cells formed only extremely sparse and slow-growing colonies in soft agar. In Fig. 4, data are shown on the effects of two cytotoxic agents, dacarbazine (DTIC) and cisplatin (cis-diammine-dichloroplatinum), on the viability of the pssXF-BCL2-AS or pssXF-BCL2-S cells as a function of time. In previous experiments, wild-type 518A2 cells were not chemosensitized after down-regulation of Bcl-2 protein expression by an siRNA targeted to the Bcl-2 mRNA (12). Similarly, in these experiments in which Bcl-2 expression has been permanently down-regulated, little or no chemosensitization, as assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, was produced after 3 days (Fig. 4) or even 4 days. In fact, at 4 days, the as32 clone is somewhat less sensitive to cisplatin than the pssXF-BCL2-S clones, but this is not reproduced in the as44 clone, and even in the as32 clone is seen only at two of the lower concentrations.

The induction of apoptosis by G3139 in tissue culture is independent of Bcl-2 protein expression

In previous work (12), we showed that melanoma cells undergo apoptosis in vitro after treatment with G3139 to an
equal extent in the presence or absence of Bcl-2 protein. However, in those experiments, Bcl-2 protein expression was only transiently decreased (6 days) by treatment with an siRNA. In contrast, in these experiments, Bcl-2 protein expression was stably and permanently decreased by two distinct methods.

It has also previously been shown that subsequent to the activation of the intrinsic pathway of apoptosis, cleavage of procaspase-3 to caspase-3 (17 and 19 kDa fragments) can be observed, in addition to the cleavage of poly(ADP-ribose) polymerase-1 to an 85 kDa product. After treatment with G3139, both the s23 and as32 (pssfX-transfected) cells begin to undergo apoptosis at 9 hours after the initiation of the G3139 transfection [100 nmol/L/Lipofectamine 2000 (1.9 μg/mL)], as evidenced by the formation of the caspase-3 and poly(ADP-ribose) polymerase-1 cleavage products. [In our previous work (18) with 518A2 melanoma cells, we have shown that the emergence of these cleaved caspase-3 species directly correlates with caspase-3 activity, as measured by the production of cleaved chromogenic substrates.] Apoptotic cleavage of procaspase-3 and poly(ADP-ribose) polymerase-1 also occurs, but not quite to the same extent, with G4126, which is a two-base mismatch of G3139. Even 24 hours after the initiation of the treatment with G3139, the extent of activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase-1 are essentially identical in the sense and antisense clones (Fig. 5A). Similarly, in both the B10 (anti-Bcl-2 pcDNA3.1-shRNA) and M10 (empty vector) clones, activation of caspase-3 and poly(ADP-ribose) polymerase-1 cleavage could be observed by 9 hours after the initiation of G3139 treatment (Fig. 5B). During this time, the differences in Bcl-2 expression between the B10 and M10 clones did not change. Therefore, as previously shown, the induction of apoptosis by G3139 in 518A2 melanoma cells is independent of Bcl-2 protein expression in vitro.

**Release of cytochrome c from mitochondria treated with G3139**

Bcl-2 is a mitochondrial membrane protein that is believed to bind to the VDAC. We have shown that G3139 can interact with VDAC (16) and that this interaction leads to VDAC closure (i.e., loss of ion conductance). At the same time as VDAC closure occurs, cytochrome c is released from the mitochondrial intermembrane space (16). Cytochrome c then binds to Apaf-1, dATP, and procaspase-9 in the apoptosome; this complex cleaves procaspase-3 to caspase-3 and initiates the intrinsic apoptotic pathway (25–27). As shown in Fig. 6, the release of cytochrome c could be detected to a similar extent in samples of isolated mitochondria from either pssfX-BCL2-AS or pssfX-BCL2-S after treatment with 5 to 40 μmol/L of G3139. These data show that the G3139-induced release of cytochrome c is also substantially Bcl-2 independent in tissue culture.

**518A2 melanoma cell xenografts in severe combined immunodeficient mice**

The down-regulation of the expression of Bcl-2 protein persists for as long as 21 days after injection into mice, as determined by Western blotting of an excised tumor (Fig. 7A). The rate of growth of the pssfX-BCL2-AS– and B10-transfected cells, both of which have greatly diminished expression of Bcl-2 protein, are virtually identical in vitro (not shown). Essentially, after the initial injection of the cells into the flanks of the animals, tumors in ~50% of the animals will grow for ~7 to 10 days. In the remainder, however, with either of the Bcl-2 suppressed clones, no tumor growth could be observed. Furthermore, those tumors that did grow reached a maximum volume of only ~50 to 100 mm³, at which point they regressed in all animals, and could not be found at all in the majority of the animals at necropsy. These results are in dramatic contrast to what was observed with the xenografted pssfX-BCL2-S or M10 clones. In this case, as shown in Fig. 7B, tumors grew to a large size in virtually all animals injected, requiring the eventual sacrifice of the animals. Comparisons of the growth rate between pairs of Bcl-2 knockdown tumors versus controls were made with the Mann-Whitney test \[P = 0.0002, \text{pssfX-BCL2-AS versus pssfX-BCL2-S}; P = 0.0019, \text{B10 versus M10 (two-tailed)}\]. These in vivo results are dramatically different than what had previously been observed in tissue culture.
Discussion

The vector-generated expression of ssDNA in cells, as first described by Chen et al. (20), helps to ameliorate some of the problems inherent in the use of antisense oligonucleotides for silencing gene expression, including their relatively transient activity and substantial toxicity. The ssDNA expression system employed in this work, or its prototypes, has been successfully used to down-regulate the intracellular expression of C-raf via production of a DNAzyme (19, 20), to generate a 34-mer G-rich oligomer capable of triplex formation (28), and to produce a quadruplex-forming 29-mer G-rich species (29) that has been shown to have significant antiproliferative activity (30, 31). In this work, stable transfection of 518A2 melanoma cells with the pssXF-BCL2-AS plasmid produced two clones (as32 and as44) in which Bcl-2 protein and mRNA expression was significantly reduced (95-97%). However, as expected based on our previous work (7), down-regulation of Bcl-2 expression in the clones did not produce chemosensitization to standard cytotoxic agents. Further, the apoptosis induced by G3139 from isolated mitochondria was similar in the wild-type, sense (pssXF-BCL2-S), and antisense (pssXF-BCL2-AS) transfectants was Bcl-2 independent. In addition, the ability of G3139 to induce release of cytochrome c from isolated mitochondria was similar in the sense and antisense clones.

To verify the results obtained from Bcl-2 silencing by the ssDNA methods, we employed an entirely different silencing process and found that the in vivo results were identical. The use of expression cassettes to produce shRNAs has been well documented (see refs. 32–36), although questions of specificity certainly remain. The intracellular expression of shRNAs has the distinct advantage over chemically synthesized siRNAs (37) of providing long-term (i.e., months) stable gene silencing in continuously passaged cell lines.

The plasmid employed in this work was designed by the method of Castanotto and Scherer (21). It contains the TTGTGTAG loop sequence, which these authors have found to be effective for shRNA processing compared with other loops. The 3′ and 5′ two nucleotides of the transcribed shRNA loop sequence (UUUUGUGUGAG) can potentially form RNA-RNA intraloop base-pairing interactions (rG:rU and rA:rU). In the event that any of these two nucleotides remain within the antisense sequence after Dicer cleavage of the double-stranded RNA (due to the short length of the original target site), they can pair with the two nucleotides following the target sequence (see Materials and Methods). The resulting antisense guide sequence from the shRNA could have up to 22 nucleotides of complementarity with the Bcl-2 gene, with the original target site extended one nucleotide at its 5′ end and, at most, two nucleotides at its 3′ end. Nevertheless, our data show that this design was effective in down-regulating the Bcl-2 transcripts in transfected cells.

Therefore, although Bcl-2 expression is only a very minor modulator of apoptosis and/or chemosensitization in 518A2 melanoma cells in the in vitro setting, this does not seem to be the case in vivo. Xenografted cells that lack Bcl-2 expression may...
either grow to form small tumors that either rapidly regress or do not grow at all. What could be the reason for this? Iervolino et al. (38) have developed evidence that Bcl-2 overexpression in the M14 human melanoma cell line increases the hypoxia-induced expression of vascular endothelial growth factor protein and mRNA and the stabilization of the vascular endothelial growth factor mRNA. The expression of the hypoxia-inducible factor 1α transcription factor, a positive regulator of vascular endothelial growth factor gene expression, was also up-regulated. Similar results were also observed in a breast cancer line (39). However, in our transfected melanoma cells, down-regulation of vascular endothelial growth factor protein expression was not observed by Western blotting (not shown). Up-regulation of Bcl-2 protein expression in melanoma cells has also been postulated to increase their aggressiveness via up-regulation of matrix metalloproteinase-2 and other matrix metalloproteases (40). However, we did not observe down-regulation of matrix metalloproteinase-2 or matrix metalloproteinase-9 activity after zymography of conditioned media from the transfectants versus the controls (not shown). Conditioned medium from Bcl-2-transduced microvascular endothelial cells was found to promote neovascularization in a rat corneal assay and was attributed to the Bcl-2-induced up-regulation of the proangiogenic molecules IL-8 and CXCL1 (41), which was probably mediated through the NF-κB pathway. We had therefore postulated that the striking differences in the in vivo growth rates between the sense and antisense clones were due to the ability of Bcl-2 to act as a proangiogenic protein. However, after sectioning and staining of the pssXF-BCL2-S xenograft tumors, extensive necrosis in the setting of very poor vascularity was observed (not shown), vitiating any possible histologic comparison with whatever pssXF-BCL2-AS xenografted tumors could be obtained. Therefore, the precise reasons that the down-regulation of Bcl-2 protein expression inhibit xenograft growth are presently unknown.

Finally, the results obtained from the xenografts in which Bcl-2 expression has been silenced are reminiscent of the clinical melanoma setting in which patients present with metastatic disease but in whom no primary lesion can be located. It is possible, although speculative, that this may depend on the levels of Bcl-2 in the primary lesion: primary melanomas lacking sufficient Bcl-2 may be able to grow to a certain size but will then regress, similar to some of the melanoma xenografts examined in this work. On the other hand, those melanoma daughter cells that have evolved with up-regulated Bcl-2 expression can continue to grow and form very large tumors.

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Bcl-2 Protein in 518A2 Melanoma Cells *In vivo* and *In vitro*

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