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Impact of Targeting the Adenine- and Uracil-Rich Element of *bcl-2* mRNA with Oligoribonucleotides on Apoptosis, Cell Cycle, and Neuronal Differentiation in SHSY-5Y Cells

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ABSTRACT

We have identified previously a destabilizing adenine- and uracil-rich element (ARE) in the 3′-UTR of *bcl-2* mRNA that interacted with ARE-binding proteins to down-regulate *bcl-2* gene expression in response to apoptotic stimuli. We have also described three contiguous 2′-*O*-methyl oligoribonucleotides (ORNs) in both sense and antisense orientation with respect to the *bcl-2* ARE that are able to regulate the *bcl-2* mRNA half-life and Bcl-2 protein level in two different cell lines. Here we show that treatment of neuronal cell line (SHSY-5Y) with antisense ORNs targeting the *bcl-2* ARE (*bcl-2* ARE asORNs) prevents *bcl-2* down-regulation in response to apoptotic stimuli with glucose/growth factor starvation (Locke medium) or oxygen deprivation and enhances the apoptotic threshold as evaluated by time-lapse videomicroscopy, fluorescence-activated cell sorting analysis, and caspase-3 activation. Additional effects of *bcl-2* ARE asORNs included inhibition of cell cycle entry and a marked increase of cellular neurite number and length, a hallmark of neuronal differentiation resulting from *bcl-2* up-regulation. The ability of *bcl-2* ARE asORNs to enhance the apoptotic threshold and to induce neuronal differentiation implies their potential application as a novel informational tool to protect cells from ischemic damage and to prevent neuronal degeneration.

Regulation of apoptosis is of vital importance for adult organisms to maintain the numeric homeostasis of cell populations or to eliminate damaged cells. Consequently, pathogenesis of a wide variety of human diseases is frequently related to apoptosis impairment. For instance, tumors and autoimmune diseases result very often from defective apoptosis. Likewise, excessive apoptosis plays a key role in the pathogenesis of numerous diseases, including neurodegenerative disorders, such as multiple sclerosis, Alzheimer's, Parkinson's, or Huntington's disease, or pathological conditions, in particular ischemia, affecting different tissues. *Bcl-2* expression is a general mechanism of resistance to apoptosis that acts at various locations inside the cell (Annis et al., 2004). To undergo apoptosis, cells need to switch-off the *bcl-2* gene expression (Haldar et al., 1994). Therefore, preventing *bcl-2* down-regulation could be, at least in principle, a very suitable strategy to treat diseases characterized by excessive apoptosis and, particularly in the neurodegenerative pathologies, to promote conditions that could slow down neuron loss (Belcredito et al., 2001; Yi et al., 2006). Furthermore, stabilizing *bcl-2* expression could rescue cells committed to apoptosis by hypoxia/ischemia-related stress (Cao et al., 2002). Recently, simvastatin has been found to protect neurons from excitotoxicity by up-regulating *bcl-2* mRNA and protein by a still-to-be-clarified mechanism (Johnson-Anuna et al., 2007).

Differentiation and cell cycle are other cellular programs in which *bcl-2* plays a role. Evidence indicates that *bcl-2* expression is involved in promoting and accelerating neuronal dif-

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ABBREVIATIONS: AU, adenine and uracil; ARE, adenine and uracil-rich element; AUBP, adenine and uracil-rich element binding proteins; ORN, 2′-*O*-methyl oligoribonucleotide; asORN, antisense 2′-*O*-methyl oligoribonucleotide; nt, nucleotide; degORN, degenerated 2′-*O*-methyl oligoribonucleotide; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DRB, 5,5-dichloro-1-β-D-ribofuranosylbenzimidazole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; CFSE, carboxyfluorescein diacetate succinimidyl ester.

ferentiation (Abe-Dohmae et al., 1993; Suzuki and Tsutomi, 1998; Eom et al., 2004). On the other hand, *bcl-2* up-regulation is also known to have an inhibitory effect on cell cycle entry independently from its antiapoptotic activity (Mazel et al., 1996; Vairo et al., 1996; Huang et al., 1997).

Increased knowledge of molecular determinants of apoptosis regulation and execution is now offering new molecular targets suitable to repair apoptosis dysfunctions (Nicholson, 2000; Hersey et al., 2006). Several antiapoptotic molecules have been proposed as therapeutics for neurodegenerative diseases (Garber, 2005). Post-transcriptional control of gene expression based on mRNA half-life or translation regulation has been known for many years for a variety of genes. A number of *cis*-acting elements are known to stabilize or destabilize the relevant mRNA, among which adenine and uracil-rich elements (AU-rich elements, AREs) located in the 3′-UTR of many mRNAs modulate mRNA stability via interaction with stabilizing or destabilizing ARE-binding proteins (AUBPs) (Bevilacqua et al., 2003; Barreau et al., 2005). The ability of AREs to interact with endogenous AUBPs has suggested that these sequences could be also accessible to exogenously vehiculated molecules (Bevilacqua et al., 2003; Luzi et al., 2003). An ARE-based mechanism of post-transcriptional control of *bcl-2* expression accounting for mRNA half-life regulation and its reduction during apoptosis has been described previously by us (Schiavone et al., 2000; Lapucci et al., 2002; Donnini et al., 2004).

In a previous work, in an attempt to stabilize the *bcl-2* mRNA by hampering its degradation machinery, we have targeted the ARE of *bcl-2* mRNA with peculiar 2′-*O*-methyl oligoribonucleotides (ORNs) in antisense orientation (Ghisolfi et al., 2005). Unlike standard DNA oligonucleotides, which form DNA/RNA heteroduplexes that can be cleaved by the RNase H (Schiavone et al., 2004), RNA oligonucleotides form stable RNA/RNA homoduplexes that are not cleaved by the RNase H (Schiavone et al., 2004). These *bcl-2* ARE-targeting antisense ORNs (*bcl-2* ARE asORNs), transported by cationic lipids into neuroblastoma and hematopoietic cell lines (i.e., SHSY-5Y and HL60, respectively), stabilized *bcl-2* mRNA and increased the level of Bcl-2 protein in a dose-dependent manner. The effect was confirmed in cell-free experiments evaluating mRNA decay. In a second work, the same effects were obtained with oligoribonucleotides homologous to the *bcl-2* ARE and therefore were acting as decoy-aptamers (Bevilacqua et al., 2007).

Here we demonstrate that *bcl-2* ARE asORNs were able to inhibit apoptosis by partially preventing the degradation of *bcl-2* mRNA and consequently the reduction of Bcl-2 protein levels in response to apoptotic stimuli. Apoptosis inhibition was accompanied by neuronal differentiation, as evaluated by counts of neurites and cell morphology, and inhibition of cell proliferation. These effects render *bcl-2* ARE asORNs as potential candidates for pharmacological interventions, particularly in the field of ischemic stress and neurodegenerative diseases.

Materials and Methods

Cell Cultures and Transfection. SHSY-5Y cell line, a neuronal subline of bone marrow biopsy-derived line SK-N-SH of human neuroblastoma, was purchased from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK) and maintained in Ham's F12/minimal essential medium (1:1) supplemented with 5% fetal bovine serum, 1% nonessential amino acids, and 1% glutamine in 100% humidity, 37°C, and 5% CO_2 atmosphere. Transient transfections of SHSY-5Y cells with plasmid pCΔJ-bcl-2 or pCΔJ-SV2 empty vector (Tsujimoto, 1989) were carried out with Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions 48 h before treatment with the oligonucleotides described below.

2′-*O*-Methyl Antisense Oligonucleotides. Three synthetic 26- to 27mer chemically modified asORNs sequentially overlapping the *bcl-2* ARE asORN1 from 1020 to 994 nt, asORN2 from 993 to 967 nt, and asORN3 from 966 to 943 nt of b-RNA (GenBank number M14745) were synthesized and polyacrylamide gel electrophoresis-purified by Dharmacon (Chicago, IL) as described previously (Ghisolfi et al., 2005). A degenerated 25-mer oligoribonucleotide (degORN) was used as control (Ghisolfi et al., 2005). The sequences of the asORNs are the following: asORN1 (27-mer), 5′-UGUCUUAAAUAAAUAAAUCUUUUUUUC-3′; asORN2 (26-mer), 5′-UUAAUAAUGUAAAAAAUAAAUGAUAU-3′; asORN3 (26-mer), 5′-UUCCCUUUGGCAGUAAAUAGCUGAUU-3′; and degORN (26-mer), 5′-NNNNNNNNNNNNNNNNNNNNNNNNNN-3′, where N is any nucleotide. ORNs were 2′-*O*-methyl-modified, which increased their stability with respect to the natural RNA derivatives (Yoo et al., 2004). ORN cellular uptake and stability were increased by their vehiculation with the cationic lipid DOTAP (Roche, Mannheim, Germany), used as lipofection reagent, as reported previously (Luzi et al., 2003; Ghisolfi et al., 2005).

Treatments. SHSY-5Y cells were seeded at a density of 5×10^5 cells/60-mm dish 1 day before lipofection with ORNs. The lipofection mixtures were prepared by mixing 1 mM DOTAP (Roche Diagnostics) with 1 mM concentration of either the three *bcl-2* ARE asORNs or the degORN and incubated at room temperature for 20 min before adding to cells as described previously (Capaccioli et al., 1993). The final concentration of each ORN was 0.5 μM. Apoptosis was induced 3 days after ORN lipofection either by replacing the culture medium without serum and glucose, namely Locke medium (Martínez de la Escalera et al., 1992), or by culturing cells in a hypoxic atmosphere (i.e., containing 1% oxygen). The transcriptional block was obtained by treating cells with the transcription blocker 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma-Aldrich, St. Louis, MO) at 20 μg/ml added at hour 3 after application of apoptotic stimuli.

Total RNA Extraction and Real-Time Reverse-Transcriptase Polymerase Chain Reaction. After treatments, total cellular RNA was extracted from approximately 10^6 cells collected at 0, 30, 60, 120, 180, 240, and 360 min after induction of apoptosis. The RNeasy Mini Kit (Qiagen, Valencia, CA) was used according to the manufacturer's instructions. In brief, total RNA isolated from SHSY-5Y cells lipofected with asORNs or degORN was treated with RNase-free DNase (Invitrogen) and analyzed spectroscopically and by gel electrophoresis for purity and integrity, respectively. Total RNA (0.1 μg/μl) was reverse-transcribed with high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) using standard manufacturer's conditions in a total volume of 50 μl. Levels of *bcl-2* and GAPDH cDNAs of each sample were determined by quantitative real-time PCR applying TaqMan Universal MasterMix (Applied Biosystems) standard manufacturer's conditions: 5 μl of total cDNA was amplified with 2× TaqMan Universal Master Mix buffer, 20× PDAR System target bcl-2, 20× PDAR System control GAPDH, and Nuclease-Free Water (Promega, Madison, WI) to a total volume of 25 μl. Each reaction was triplicated for a better statistical reliability of results. The PCR reactions were carried out in an ABI PRISM 7000 Sequence Detection System under the following conditions: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 1 min at 60°C (40 cycles). Amplification plot and CT data were elaborated with ABI PRISM 7000 SDS Software 1.1 updated version. GAPDH data were used to normalize *bcl-2* cDNA values.

Western Blotting. The assays were performed according to standard conditions. In brief, after treatments, cells were washed twice in ice-cold phosphate-buffered saline and resuspended in 100 μl of

ice-cold RIPA lysis buffer, vortexed for 3 s, and incubated on ice for 30 min. The protein lysates were obtained by centrifugation at a high speed for 20 min at 4°C to separate nonsoluble cell debris. Proteins (20 μg/lane) were analyzed by 12% SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane (Hybond-ECL; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) in a Transblot apparatus (Bio-Rad Laboratories, Hercules, CA) at 100 V for 90 min. Blots were processed by an enhanced chemoluminescence (ECL Plus) detection kit according to the supplier's instructions (GE Healthcare). The blots were probed with a mouse monoclonal anti-Bcl-2 antibody (Upstate Biotechnology, Charlottesville, VA), rabbit polyclonal anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-caspase-3 antibody (Santa Cruz). A mouse monoclonal anti-α-tubulin antibody (Sigma-Aldrich) was used as a protein loading control.

Quantitative Evaluation of Differentiated Neurons. SHSY-5Y cells were lipofectamine-transfected with *bcl-2* ARE asORNs or the degORN as described above and cultured in 60-mm Petri dishes in growth medium. Three days after treatment, the cells were analyzed by an inverted phase contrast microscope equipped with a 10× objective and photographed by a Nikon digital camera (Nikon, Tokyo, Japan). The number of neurites was obtained as the total number of neurites per 50 cells counted independently by three researchers. The counts were performed independently for each digital image corresponding to specific treatment. The neurite length was obtained by measuring the distance between the cell nucleus and the distal part of the neurite (Aruga Mikoshiba, 2003).

Evaluation of Cell Growth. SHSY-5Y cells were lipofected with *bcl-2* ARE asORNs or degORN control as described above and cultured in 100-mm Petri dishes in growth medium. Each day during a 5-day period, cells were detached and evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) colorimetric assay (van de Loosdrecht et al., 1994).

Evaluation of Cell Proliferation by Fluorescence-Activated Cell Sorting Analysis. The number of cell divisions was assessed with the carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) assay and evaluated by fluorescence-activated cell sorting analysis (BD Biosciences, San Jose, CA). CFSE covalently binds cellular components, yielding a fluorescence (measured by flow cytometry) that is divided equally between daughter cells at each division (Lyons et al., 2001) and allows for calculating successive rounds of replication. Confluent cultures of SHSY-5Y cells were starved for 14 h in the growth medium described above but containing only 0.5% fetal bovine serum. Cells were then harvested and labeled for 10 min with 10 μM CFSE at 37°C and washed twice with culture medium. Labeled cells were plated (6×10^4/60-mm dish) and lipofected with asORNs or degORN the following day as described above. An aliquot of freshly labeled cells was used to measure the starting fluorescence level (day 0). Cells were harvested on day 5 to determine a decrease in fluorescence. These values were used to calculate the number of replicative rounds elapsed from day 0 in response to treatments (ModFit LT for Macintosh, Proliferation Protocol; Verity Software House Inc., Topsham, ME).

Evaluation of Apoptotic Events. After cell transfer to Locke medium, apoptotic events were counted by the time-lapse videomicroscopy using a Zeiss inverted phase-contrast microscope (Carl Zeiss Inc., Thornwood, NY) equipped with a 10× objective, Panasonic charge-coupled device cameras, and JVC BR9030 time-lapse video recorders as reported previously (Luzi et al., 2003). An apoptotic event was scored at the moment the cell became fully contracted and fragmented in apoptotic bodies. Apoptotic cells were also evaluated by flow cytometry with GUAVA Personal Cell Analysis System with Guava Nexin Assay (GUAVA Technologies, Hayward, CA) that uses Annexin V-PE to detect phosphatidyl serine on the external membrane of apoptotic cells. The cell-impermeant dye 7-aminoactinomycin D is included in the kit as an indicator of membrane structural integrity and for the differential assessment of the Annexin V-reactive cells into the early and late stages of apoptosis. 7-aminoactinomycin D is excluded from live, healthy cells and early apoptotic cells but permeates late-stage apoptotic and dead cells. The assay was performed according to the manufacturer's instructions.

Statistical Analysis. The statistical evaluation of the data was performed with the two-tailed Student's *t* test for unpaired values. Differences were considered statistically significant when $p \leq 0.05$. The data are reported as a percentage of the maximal value.

Results

The molecular and phenotypic effects of *bcl-2* ARE-targeting asORNs in response to apoptotic stimuli were analyzed in SHSY-5Y neuronal cells lipotransfected with asORNs or control degenerated ORN (degORN) at the concentration of 0.5 μM established previously to be the most effective (Ghisolfi et al., 2005) 3 days before the application of apoptotic stimuli. Apoptosis was induced by culturing cells either in Locke minimal medium, mimicking condition of glucose/growth factor deprivation, or in condition of hypoxia.

***Bcl-2* ARE asORNs Markedly Attenuated the Decrease of *bcl-2* mRNA Half-Life and Bcl-2 Protein Level in Response to Growth Factor Deprivation.** Transfection of the SHSY-5Y cell line with *bcl-2*-specific asORNs resulted in Bcl-2 protein increase as shown in Fig. 1, whereas no effect was observed on expression of Bax, a proapoptotic member of the bcl-2 family.

The effect of *bcl-2* ARE asORNs on *bcl-2* mRNA stability after glucose/growth factor deprivation as an apoptotic stimulus (Locke medium) in SHSY-5Y is shown in Fig. 2. *Bcl-2* mRNA stability was evaluated by quantification of *bcl-2* mRNA levels at various times after application of a transcriptional block (20 μM DRB added 3 h after transfer to

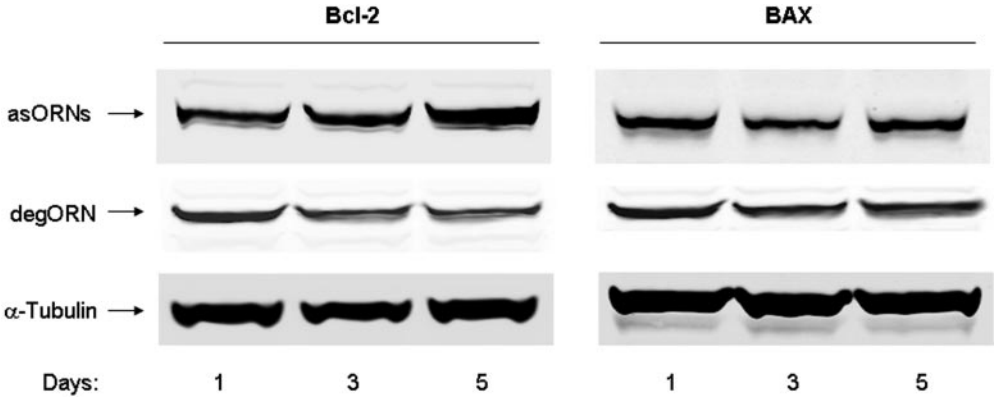


Fig. 1. Molecular effect of *bcl-2* ARE asORNs. Cells were lipofected with 0.5 μM asORNs or the degORN and 10 μM DOTAP. At the indicated times, cells were collected, and protein extracts were prepared for the Western blot analysis of Bcl-2 and Bax expression. α-Tubulin provided the loading control.

Locke medium). The *bcl-2* mRNA half-life decreased from 3 h in conventional medium to 30 min in the Locke medium. The addition of asORNs to the Locke medium counteracted the decrease, increasing the *bcl-2* mRNA half-life from 30 min to 2 h. The degORN used as control did not elicit any effect on the mRNA levels. This indicates that asORNs are endowed with strong stabilizing activity toward *bcl-2* mRNA also in condition of apoptotic stress.

We then evaluated the effect of asORNs on Bcl-2 protein levels by Western blot analysis in response to Locke medium (Fig. 3). On the third day of culturing in the Locke medium, the Bcl-2 protein levels underwent a marked decrease in SHSY-5Y cells with respect to controls cultured in normal medium (Fig. 3A). The addition of asORNs to the Locke medium markedly attenuated this decrease, whereas the addition of degORN did not have significant effects. Histograms in Fig. 3B were obtained by the densitometric analysis of bands in Fig. 3A and are means of three independent experiments.

Bcl-2 ARE asORNs Dramatically Lowered the Number of Apoptotic Cells in Response to Locke Medium or to Hypoxic Condition. The possibility that inhibition of *bcl-2* down-regulation in response to apoptotic stimuli induced by the *bcl-2* ARE asORNs could lead to enhancement of the apoptotic threshold has been evaluated. The number of apoptotic events occurring in SHSY-5Y cells cultured either in the Locke medium or in the condition of hypoxia and lipofected either with asORNs or with degORN was quantified by time-lapse videomicroscopy (Fig. 4). An apoptotic event was scored at the moment the cell detached from the substrate, shrank, and blebbed. The SHSY-5Y cells cultured in normal growth medium served as untreated control. As shown in Fig. 4A, *bcl-2* ARE asORNs but not degORN dramatically reduced the number of apoptotic events occurring in SHSY-5Y cells cultured in the Locke medium.

The protective effect of *bcl-2* ARE asORNs against apoptosis of SHSY-5Y cells induced by hypoxia was evaluated by flow cytometry with Annexin V-PE assay. The cells were maintained in the hypoxic condition for up to 48 h. As shown in Fig. 4B, already at hour 12, the hypoxia induced massive mortality (less than ~5% of viable cells) compared with controls cultured in standard atmosphere. Treatment with *bcl-2* ARE asORNs markedly counteracted this effect, protecting cells from apoptosis: the percentage of viable cells in hypoxic conditions was substantially at the level of untreated controls at hour 12 and decreased to ~80 and ~50% of viable cells at the hours 24 and 48, respectively. Treatment with the degORN did not have any significant effect on the viability compared with the untreated control.

Bcl-2 ARE asORNs Inhibited Caspase-3 Activation in Response to Locke Medium. Caspase-3 has been reported to activate death effector molecules resulting in the fragmentation of genomic DNA in association with structural and morphological changes characteristic of apoptosis. Caspase-3 is initially present as a proenzyme of 32 kDa that is cleaved in the cell undergoing apoptosis into its enzymatically active form composed of two subunits of 17 (p17) and 11 kDa (p11). Activity of the caspase-3 in SHSY-5Y cells lipofected with *bcl-2* ARE asORNs or with degORN was evaluated by Western blot analysis after 0, 24, 48, and 72 h in Locke medium (Fig. 5). The activity of caspase-3 in SHSY-5Y cells maintained in normal growth medium provided untreated control. As expected, the cleaved 17-kDa active caspase-3 subunit, undetectable in untreated controls, was evident in SHSY-5Y cells cultured in Locke medium but was markedly lowered when *bcl-2* ARE asORNs were added. The degORN did not elicit any effect. The SHSY-5Y cells transiently transfected with a *bcl-2*-harboring plasmid did not show significant caspase activation in response to Locke medium with respect to mock transfected cells, untransfected cells, and degORN-transfected cells.

Bcl-2 ARE asORNs Inhibited Cell Proliferation. *Bcl-2* expression has been reported previously to markedly diminish cell proliferation by preventing quiescent cells from reentering the cell cycle (Borner, 1996; Vairo et al., 1996; Huang et al., 1997). We have evaluated the possibility that

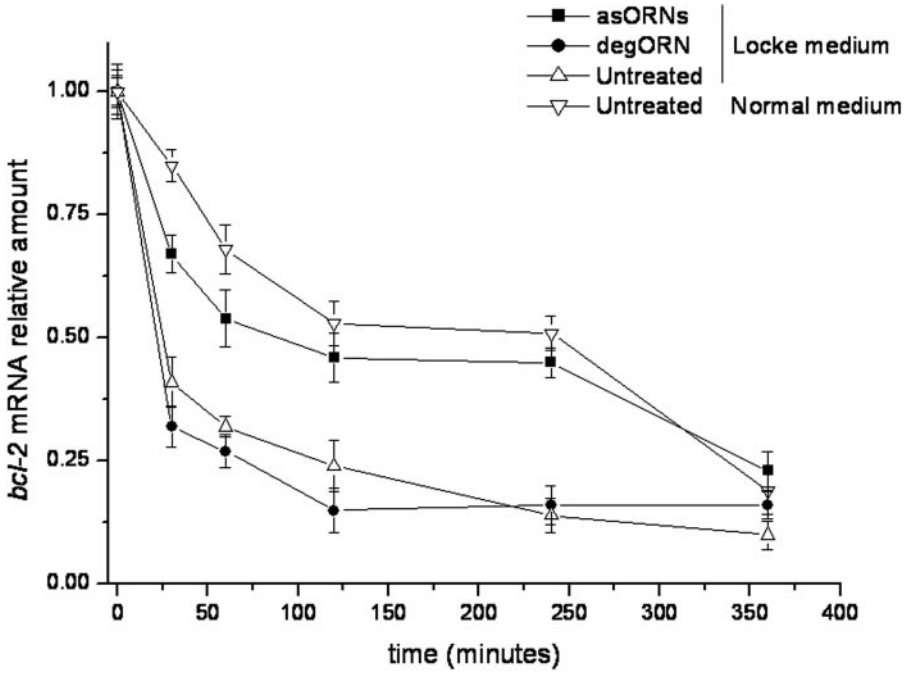


Fig. 2. Determination of *bcl-2* RNA half-life in glucose/growth factor-deprived cells (Locke medium) treated with *bcl-2* ARE asORNs. Cells were maintained as in Fig. 1 3 days before application of the apoptotic stimulus (Locke medium). Transcriptional block was obtained by adding 20 μM DRB3 h after transfer to Locke medium. At the indicated times after DRB transcriptional block, cells were collected, total RNA was extracted, and *bcl-2* mRNA was analyzed by quantitative real-time reverse-transcriptase polymerase chain reaction. Results were normalized to β-actin cDNA. Each point is the mean ± S.E. of three independent experiments. ***, $p \leq 0.001$ asORNs versus degORN or untreated at 30, 60, 120, and 240 min.

bcl-2 ARE asORNs affect cell proliferation by MTT assay and by flow cytometry (Fig. 6). The cell proliferation was evaluated by analysis of cell viability every day for 5 days with the MTT assay. The *bcl-2* ARE asORNs markedly reduced the rate of SHSY-5Y cell proliferation compared with untreated or degORN-treated controls (Fig. 6A). The distribution of cell divisions in *bcl-2* ARE asORN-treated SHSY-5Y cells compared with untreated or degORN-treated controls was determined by flow cytometry on the basis of the CFSE dye dilution (Fig. 6B). This assay allows counting the subsequent rounds of cell divisions (generations) that start from fully labeled SHSY-5Y cells on day 0. On the 5th day, approximately 70% of untreated cells approached the ninth generation, 20% of these reached the 10th generation, and only 17% were still at the eighth generation. Similar results were obtained in cells lipofected with the degORN. Instead, only 8% of *bcl-2* ARE asORN-treated cells reached the 10th generation, 50% were at the ninth generation, and 30% of cells were at the eighth generation. Furthermore, 10% of cells treated with *bcl-2* ARE asORNs were arrested at the seventh generation, demonstrating significant inhibition of SHSY-5Y proliferation rate.

***Bcl-2* ARE asORNs Induced Neuronal Differentiation.** In addition to its antiapoptotic function, *bcl-2* has been reported to have differentiating and neuroprotective properties, promoting dendrite branching and regeneration of damaged neurons. We have evaluated the ability of *bcl-2* ARE asORNs to induce neuronal differentiation in SHSY-5Y cells, assuming neuron length and number as differentiation index. The morphology of SHSY-5Y cells treated for 5 days with asORNs or the degORN or untreated controls is shown in the Fig. 7. Most asORN-treated cells assumed the differentiated phenotype, characterized by the presence of numerous relatively long and interconnected neurites (Fig. 7A). Instead,

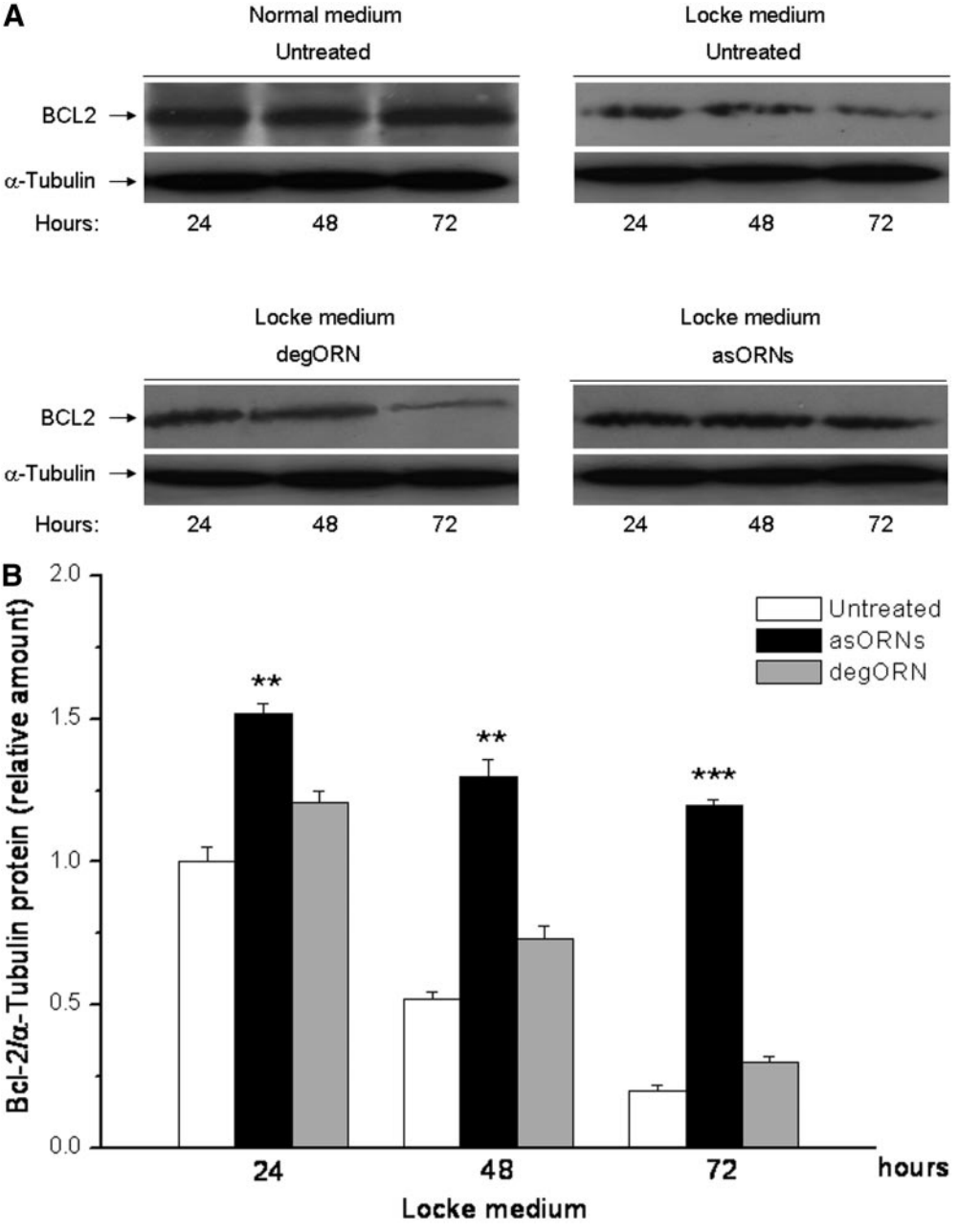


Fig. 3. Determination of Bcl-2 protein level in cells maintained in Locke medium treated with *bcl-2* ARE asORNs. a, cells were maintained and treated as in Fig. 2 except for the omission of DRB. At the indicated times, cells were collected, and proteins were extracted for the analysis of Bcl-2 by Western blot. α-Tubulin provided the loading control. The result is the representative of three independent experiments. b, densitometric histograms of relative band intensities are shown. Data are means ± S.E. of three independent experiments. **, $p \leq 0.01$ asORNs compared with degORN; ***, $p \leq 0.001$ asORNs compared with degORN.

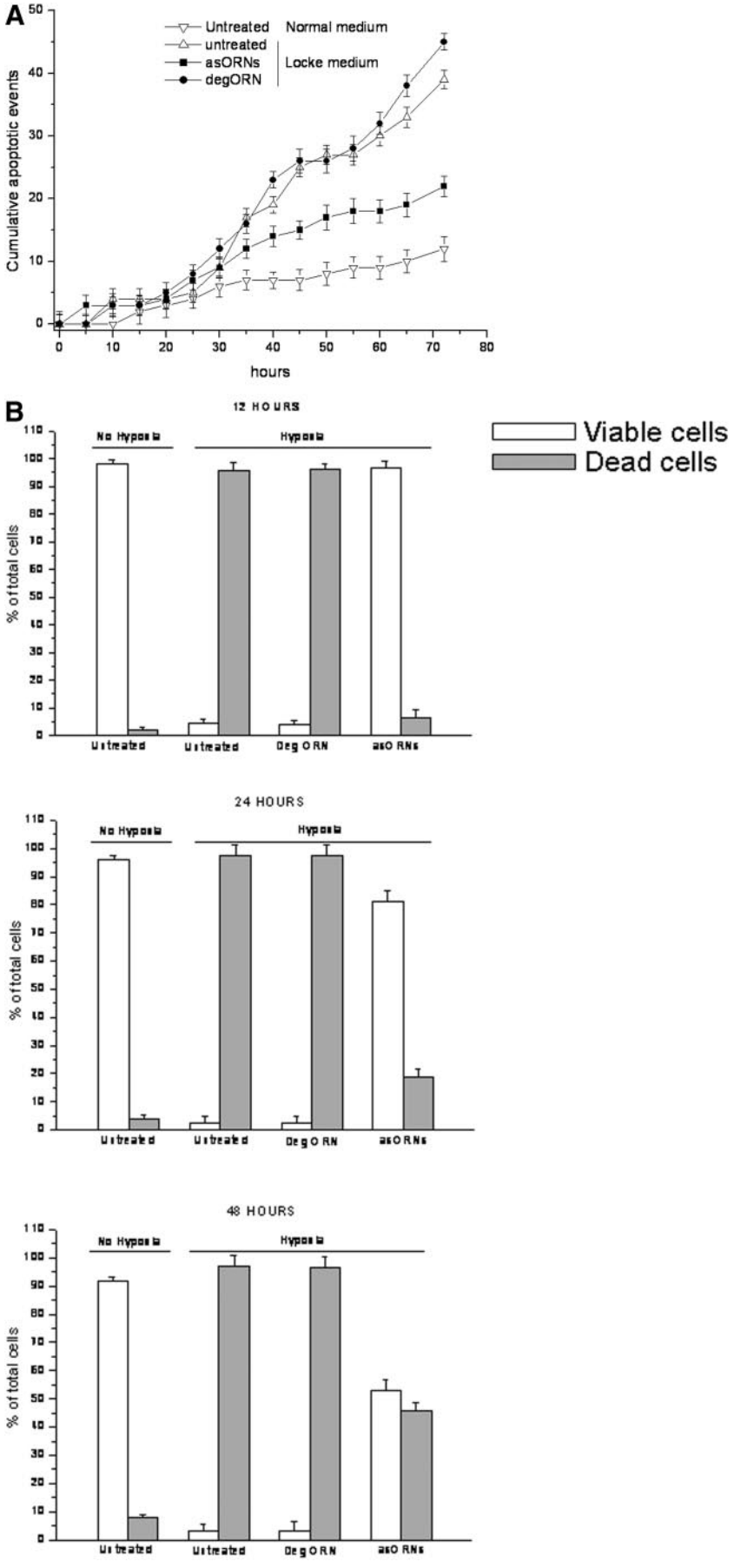


Fig. 4. Evaluation of apoptosis in cells maintained in Locke medium or in hypoxic conditions and treated with *bcl-2* ARE asORNs or degORN. a, apoptotic events in SHSY-5Y cells maintained in Locke medium after a 3-day pretreatment with asORNs (as in Fig. 3) were counted by time-lapse videomicroscopy. Apoptotic events were scored at the moment the cells were fully shrunk and apoptotic bodies appeared. Each point is the mean number of cumulative apoptotic events ± S.E. of three independent experiments. ∗, $p \leq 0.005$ Locke + asORNs versus Locke or Locke + degORN from 40 to 72 h. b, cells, maintained in hypoxic conditions for 12, 24, and 48 h and treated as above, were analyzed by flow cytometry for expression of Annexin V. Bar graphs show the compiled mean values ± S.E. of four independent experiments.

untreated cells and degORN-treated cells maintained the undifferentiated phenotype, characterized by very small number or even total absence of neurites (Fig. 7A). As expected, the transient transfection of a *bcl-2*-harboring plasmid but not of an empty vector was able to promote neurite formation in SHSY-5Y cells (Fig. 7A). The median length of neurites and the median number of neurites per cell are reported in Fig. 7B, top, and in Fig. 7B, bottom, respectively.

Discussion

The antisense strategy, aimed to specifically down-regulate gene expression, flourished in the last decades and culminated with the burst of pharmacogenomics and RNA interference. On the contrary, little attention has been given so far to strategies aimed to up-regulate the expression of target genes and/or to prevent their switching off. This is happening despite the wide number of human diseases involving inadequate expression of specific genes and the suitability of deterministic up-regulation of a given gene product to study its function, overcoming the drawbacks of gene transfection. In particular, excessive apoptosis resulting from down-regulation of *bcl-2* expression often plays a key role in the pathogenesis of several human diseases, ranging from neurodegenerations to AIDS, from atherosclerosis to ophthalmologic diseases, which suggests the breadth of potential therapeutic opportunities offered by *bcl-2* up-regulating molecular tools.

In a previous work, we have shown that three contiguous synthetic asORNs targeting the *bcl-2* mRNA regulative ARE stabilize *bcl-2* mRNA and enhance Bcl-2 protein levels in a dose-dependent fashion (Ghisolfi et al., 2005). The effectiveness of this strategy relies on the accessibility of any mRNA *cis*-acting element by the relevant transacting factors modulating the RNA decay machinery that assembles on it (Bevilacqua et al., 2003). Therefore, up-regulation of *bcl-2* gene expression by targeting its destabilizing *bcl-2* ARE with synthetic modified single-strand RNAs could be a paradigm for any gene regulated by AREs or to any other gene carrying *cis*-acting elements in their RNA.

Here we show that this innovative approach, stabilizing *bcl-2* mRNA and leading to increased Bcl-2 protein level without affecting the *bcl-2* family member Bax, in SHSY-5Y

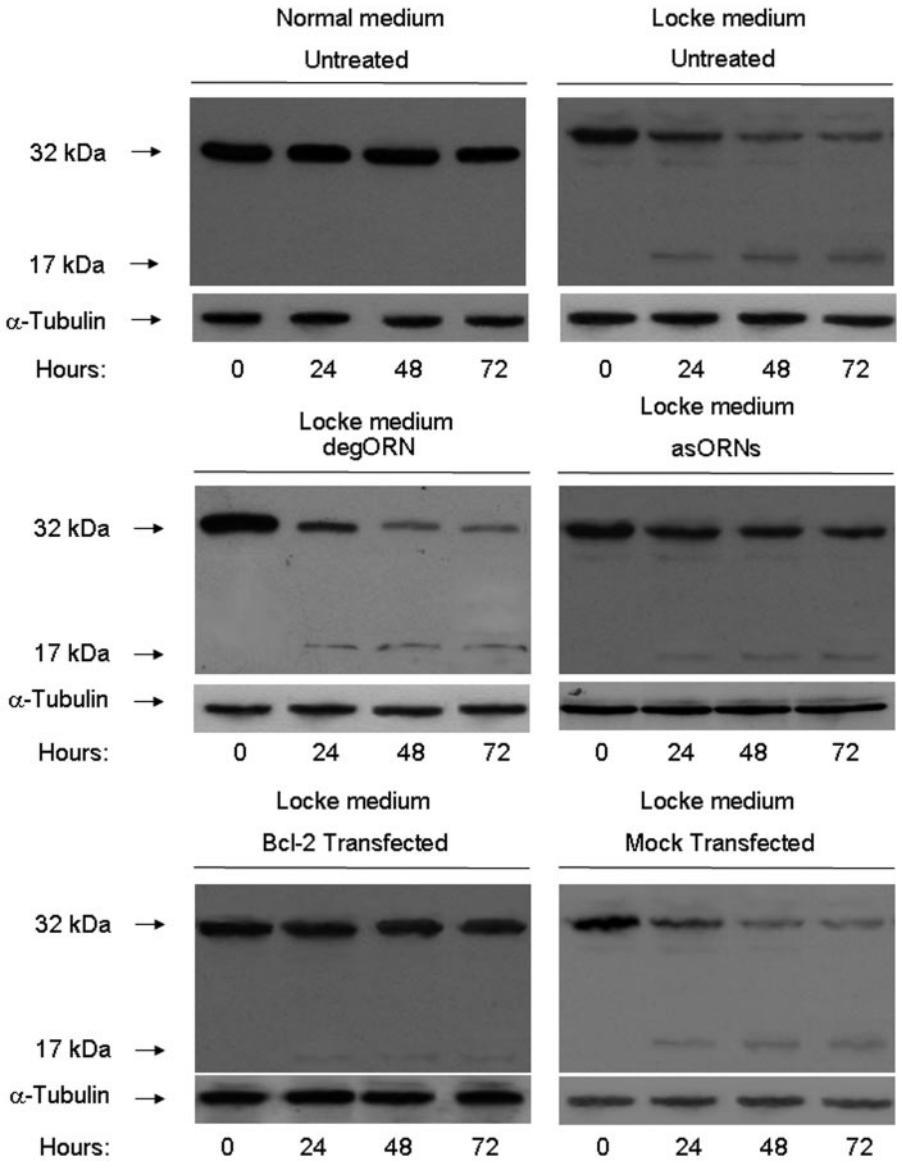


Fig. 5. Caspase-3 activation in cells maintained in Locke medium and treated with *bcl-2* ARE asORNs. Protein extracts from cells maintained and treated as in Fig. 3 and from cells transiently transfected as indicated were analyzed for the expression of the nonactivated and activated caspase-3 by Western blot. Caspase-3 activation was evaluated as decrease of the 32-kDa proenzyme band and/or increase of bands corresponding to the enzymatically active subunits of 17 (p17) and 11 kDa (p11) at the indicated times. α-Tubulin provided the loading control. Data are representative of three independent experiments.

cells, inhibits *bcl-2* gene down-regulation in response to apoptotic stimuli. As a consequence, this approach prevents apoptosis and modifies fundamental cellular programs. Indeed, the three synthetic *bcl-2* ARE asORNs protected *bcl-2* mRNA from the fast degradation triggered by the deprivation of growth factors and glucose or by hypoxic stress and maintained the Bcl-2 protein at relatively high levels in SHSY-5Y cells compared with degORN-treated or untreated controls. The relatively high steady-state level of Bcl-2 protein in asORN-treated cells, in line with the relatively high

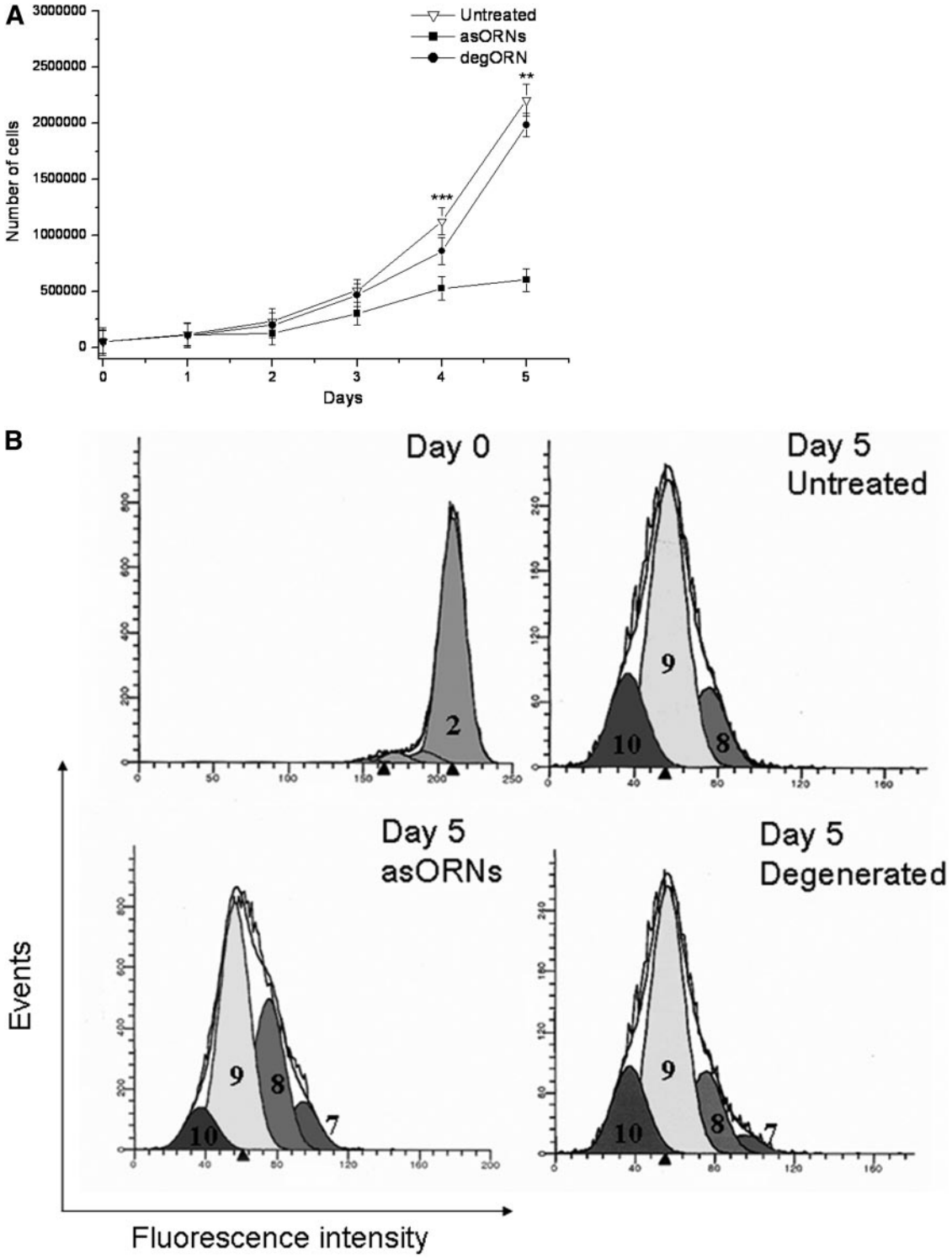
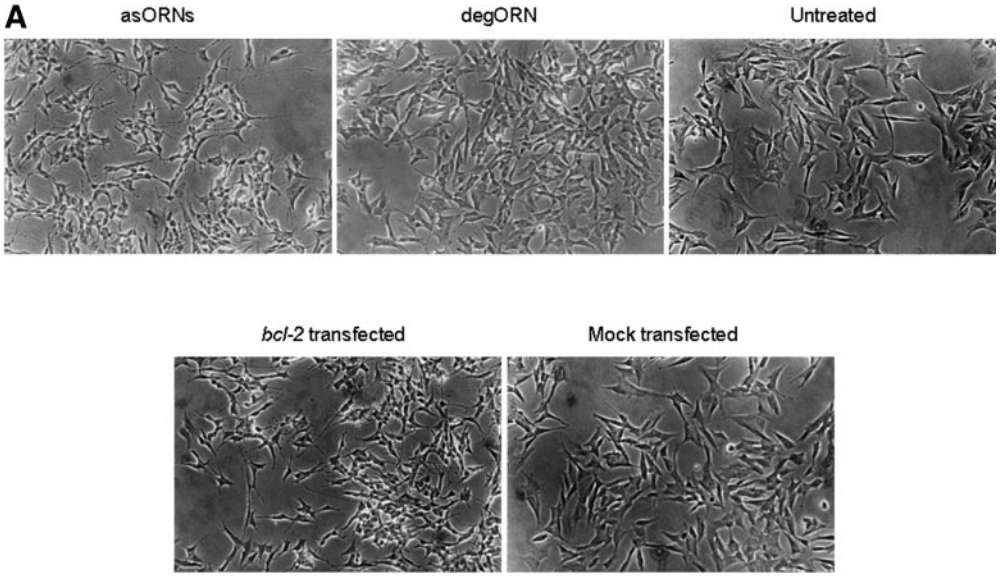


Fig. 6. Viability and proliferation rate in growth factor-deprived cells treated with *bcl-2* ARE asORNs. a, viability of cells, maintained and treated as in Fig. 2, was evaluated by the MTT colorimetric assay. Data are compiled means ± S.E. of five independent experiments. **, $p \leq 0.01$ asORNs compared with degORN or untreated at 4 days. ***, $p \leq 0.001$ asORNs compared with degORN o untreated at 5 days. b, the rate of proliferation was analyzed for CFSE fluorescence by flow cytometry on days 0 and 5 after lipofection. Fluorescence peaks decrease according to the loss of CFSE fluorescence over time as cells divide. Peak numbers correspond to cell division/generation's number. Values are representative of five independent experiments.

bcl-2 mRNA level (Ghisolfi et al., 2005), counteracted activation of apoptotic program. Counteraction of apoptosis was maximal in the initial phases of the apoptotic program but was still apparent at 48 h.

Although the main function of Bcl-2 protein is to increase the apoptotic threshold of the cells, Bcl-2 has also another fundamental activity that is the inhibition of cell proliferation. Moreover, in proper settings, the above biological property of Bcl-2 can be associated with the activation of the differentiation program (Grossmann et al., 2000). Both effects have been obtained in the SHSY-5Y neuronal cell line as a result of Bcl-2 overproduction induced by treatment with *bcl-2* ARE asORNs or by transient transfection of a *bcl-2* harboring plasmid. Clearly, *bcl-2* ARE asORNs reduced cell number without affecting cell viability and lowered proliferative kinetics compared with untreated controls. It is most



B

Samples	n° of neurites/50 cells	Neurites/cell
Untreated	13	0.26
degORN	16	0.31
asORNs	48	0.96
bcl-2 transf.	60	1.10
Mock	20	0.49

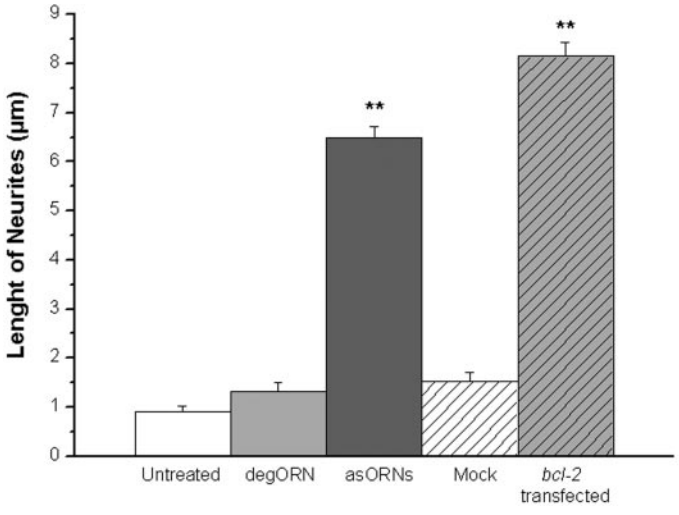


Fig. 7. Neuronal differentiation induced by *bcl-2* ARE asORNs. a, cells were treated with asORNs, degORN, transiently transfected as indicated, or untreated, respectively, and analyzed microscopically for sprouting of neurites. Scale bar, 10 μm. b, the number of neurites per cell and their length were calculated as indicated under *Materials and Methods*. Data are the means ± S.E. of five independent experiments. **, $p \leq 0.001$ asORNs compared with degORN or untreated.

relevant that *bcl-2* ARE asORNs induced neuronal cell differentiation evaluated on the basis of the length of neurites and their number per cell, albeit at a lower degree than exogenously expressed *bcl-2*. The impact of synthetic single-strand RNAs complementing the *cis*-acting AU-rich element of *bcl-2* mRNA on proliferation and differentiation of human cells suggests the high pharmacological potential of an innovative strategy able to modify cellular programs by acting at the post-transcriptional level of the gene expression.

The hypothesis that short *bcl-2* ARE-targeting single-strand RNAs could modulate *bcl-2* expression was in part inspired by our discovery in t(14;18) lymphoma cells that a hybrid *bcl-2*/IgH antisense transcript caused *bcl-2* overexpression by overlapping the *bcl-2* ARE of the *bcl-2*/IgH mRNA (Capaccioli et al., 1996). This hypothesis is strongly supported by evidences obtained by Meisner et al. (2004), analyzing the effects of synthetic ORNs on the secondary structure and stability of ARE-controlled transcripts. They demonstrated in peripheral blood mononuclear cells that ARE accessibility of synthetic ARE-controlled transcripts (tumor necrosis factor-α and interleukin-2) by the stabilizing AUBP HuR can be either opened or closed by computationally designed ORNs. This property decides the fate (stabilization or decay) of the transcripts, indicating the possibility to manipulate ARE-controlled gene expression by exogenous ARE openers or closers. It is conceivable that pairing of the *bcl-2* ARE with asORNs in our cellular model could have opened the *bcl-2* ARE accessibility to stabilizing AUBPs or closed the *bcl-2* ARE accessibility to destabilizing AUBPs, or could have done both.

Short 2′-*O*-methyl-oligonucleotides designed to target a complementary region within an endogenous messenger RNA are not supposed to activate ribonuclease activities, and they are not known to induce RNA degradation by heteroduplex ribonucleases (Schmitz et al., 2001). Actually, 2′-*O*-methyl ORNs have been used to temporarily interfere with translation or other mRNA-involving processes, such as enforced exon skipping (Schmitz et al., 2001). In addition, 2′-*O*-methyl modified small interfering RNA duplexes show reduced efficacy in down-regulating gene expression, even when the modification is restricted to as few as 3 of 20 positions on the antisense strand (Prakash et al., 2005). We have shown previously that our *bcl-2* ARE asORNs mask the *bcl-2* mRNA ARE with respect to the relevant transacting AUBPs and inhibit their functions in a reversible fashion (Ghisolfi et al., 2005). Furthermore, their application to SHSY-5Y cells did not induce obvious toxic effects other than the specific action at the level of the target gene.

Despite the success of the *bcl-2* ARE asORNs in up-regulating *bcl-2* expression in a neuronal cell line, the pharmacological potential of these tools requires some general considerations. Indeed, the molecular and phenotypic effects of modified *bcl-2* expression are far from being obvious. Despite that *bcl-2* overexpression is commonly associated with tumor onset and progression, the role of Bcl-2 protein in tumors needs to be further clarified. Indeed, rapid tumor progression and bad prognosis are often paradoxically associated with loss of Bcl-2 function, an effect that could be in part explained by the ability of Bcl-2 to inhibit the cell cycle. Likewise, *bcl-2* overexpression is usually associated with unspecific drug resistance in most tumors (Kiechle and Zhang, 2002).

Furthermore, studies and even clinical trials conducted to evaluate the anticancer effectiveness of combined application of *bcl-2* down-regulating antisense molecules with conventional chemotherapeutics gave controversial results. Numerous evidences indicated a synergism between antisense oligodeoxyribonucleotide-mediated *bcl-2* down-regulation and classic anticancer compounds (Milella et al., 2004). Whatever is the role of Bcl-2 in tumors, maintaining the basal level of Bcl-2 above the threshold that triggers apoptosis execution might represent a new therapeutic strategy to treat those humans diseases in which deficient *bcl-2* expression plays a key role and to prevent tissue damages in the case of ischemic and stress conditions.

Particularly attractive is the potential application of *bcl-2* ARE asORNs to neurodegenerative conditions, in which induction of high level of Bcl-2 protein has already shown therapeutic values (Lawrence et al., 1996). The recent finding that statins are implied in neuroprotection by a bcl-2-dependent mechanism (Johnson-Anuna et al., 2007) further supports our approach. Authors suggest that transcriptional and post-transcriptional mechanisms can be involved in simvastatin-induced up-regulation of bcl-2. In particular, statins have already been demonstrated to positively modulate mRNA stability (Bonetti et al., 2003) and can be thus considered for synergic therapeutic effects.

Besides asORN-mediated up-regulation of *bcl-2* expression by the specific targeting of its ARE, this work might open a new avenue for the pharmacological enhancement of any gene expression, provided a destabilizing element is harbored on its mRNA. Besides enhancement of oncosuppressor functions in cancer gene therapy, the restoration of depressed activity of specific genes is a common requirement for the treatment of human diseases. Although oligonucleotides are still facing substantial difficulties entering into cells and explicating activity in experimental animals, the reversibility of their action and their apparent low toxicity could encourage developing a general modality to apply these molecules to positively regulate gene expression in a very specific fashion.

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