

## **Apoptosis Induction in Human Melanoma Cells by Inhibition of MEK Is Caspase-Independent and Mediated by the Bcl-2 Family Members PUMA, Bim, and Mcl-1**

Yu Fang Wang, Chen Chen Jiang, Kelly Anne Kiejda, Susan Gillespie, Xu Dong Zhang, and Peter Hersey

**Abstract Purpose:** Given that inhibitors of mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK) are being introduced into treatment for melanoma, the present study was carried out to better understand the mechanism by which they may induce apoptosis of melanoma cells.

**Experimental Design:** A panel of human melanoma cell lines and fresh melanoma isolates was assessed for their sensitivity to apoptosis induced by the MEK inhibitor U0126. The apoptotic pathways and regulatory mechanisms involved were examined by use of the inhibitor and small interfering RNA (siRNA) techniques.

**Results:** Inhibition of MEK induced apoptosis in the majority of melanoma cell lines through a mitochondrial pathway that was associated with the activation of Bax and Bak, release of mitochondrial apoptogenic proteins, and activation of caspase-3. However, apoptosis was independent of caspases and instead was associated with mitochondrial release of AIF as shown by the inhibition of apoptosis when AIF was knocked down by siRNA. Inhibition of MEK resulted in the up-regulation of the BH3-only proteins PUMA and Bim and down-regulation of the antiapoptotic protein Mcl-1. These changes were critical for the induction of apoptosis by U0126 as siRNA knockdown of PUMA or Bim inhibited apoptosis, whereas siRNA knockdown of Mcl-1 increased apoptosis particularly in the apoptosis-resistant cell lines.

**Conclusions:** Apoptosis of melanoma cells induced by the inhibition of the MEK/ERK pathway is mediated by the up-regulation/activation of PUMA and Bim and down-regulation of Mcl-1. Release of AIF rather than the activation of caspases seems to be the mediator of apoptosis. Our results suggest that cotargeting Mcl-1 and the MEK/ERK pathway may further improve treatment results in melanoma.

The treatment of metastatic melanoma by conventional chemotherapeutic and biological agents remains unsatisfactory (1). This is believed to be largely due to the resistance of melanoma cells to induction of apoptosis that is conferred by inappropriately activated survival signaling pathways (2, 3). Among them, the mitogen-activated protein kinase (MAPK) pathway has drawn increasing attention for the development of targeted therapy in melanoma because it is constitutively activated in the majority of melanomas (1–5) due to activating mutations in BRAF (6–8) or N-Ras (4, 9). Extracellular signals

from adhesion interactions or autocrine growth factors acting through receptor tyrosine kinases (9, 10) also have important roles in the constitutive activation of the pathway.

Apoptosis mediated through the mitochondrial pathway is regulated by the Bcl-2 family of proteins (11, 12). Antiapoptotic proteins such as Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 protect mitochondrial integrity, whereas the proapoptotic members of the family promote the release of apoptogenic proteins such as cytochrome *c*, Smac/DIABLO, and AIF from the mitochondria (11, 12). The proapoptotic proteins of the Bcl-2 family can be further divided into the BH3-only proteins including Bid, Bad, Bim, PUMA, and Noxa and their effectors, multidomain proteins Bax and Bak (11, 12). Activation of BH3-only proteins of the Bcl-2 family are essential in the induction of apoptosis because they act as “death ligands” to activate the proapoptotic Bax and Bak proteins by directly binding to them or by indirectly displacing them from antiapoptotic Bcl-2 family members (11, 12). The antiapoptotic Mcl-1 may also have different functions. In particular, it was proposed to play a unique apical role, elimination of which is required at an early stage of induction of apoptosis (13).

We have shown that the inhibition of MAP/extracellular signal-regulated kinase (ERK) kinase (MEK) sensitized melanoma cells to apoptosis induced by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 14). The mechanism involved was

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poorly understood but seemed to involve events upstream of the mitochondria, which resulted in enhanced activation of Bax (14). Others have suggested that MEK inhibition may dephosphorylate and activate Bad (15). In the present study, we have further examined the mechanism(s) by which inhibition of the MEK/ERK signaling pathway may induce apoptosis in melanoma cells. We report that the inhibition of MEK induces apoptosis of melanoma cells by a mitochondrial apoptotic pathway that is independent of the caspase cascade and is mediated by the up-regulation/activation of the proapoptotic BH3-only proteins PUMA and Bim and the down-regulation of the antiapoptotic Bcl-2 family member Mcl-1.

## Materials and Methods

**Cell culture and reagents.** Human melanoma cell lines Me4405, IgR3, Mel-FH, Mel-RMu, Mel-RM, Mel-CV, Sk-Mel-28, Sk-Mel-110, and MM200 have been described previously and were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories; refs. 14, 16). Melanocytes were kindly provided by Dr. P. Parsons (Queensland Institute of Medical Research, Brisbane, Australia) and cultured in medium supplied by Clonetics (Edward Kellar, Victoria, Australia). The MEK inhibitor, U0126, was purchased from Promega Corporation. The pan-caspase inhibitor Z-Val-Ala-Asp (OMe)-CH<sub>2</sub>F (z-VAD-fmk) and the mouse monoclonal antibody (MAb) against Bak (Ab-1) were purchased from Calbiochem. The rabbit polyclonal Abs against caspase-3, caspase-8, and caspase-9 were from Stressgen. The mouse MABs against Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, Bax, Bad, AIF, and phosphorylated ERK1/2 and the rabbit polyclonal Ab against Smac were purchased from Santa Cruz Biotechnology. The rabbit polyclonal Abs against ERK1/2, MEK, PUMA, and Bid were from Cell Signalling Technology. The MAb against Noxa and the polyclonal Ab against Bim were purchased from Imgenex. The mouse MABs against cytochrome *c* and PARP were from PharMingen (Bioclone). The mouse MAb against XIAP was purchased from Transduction Laboratories. The rabbit polyclonal anti-Bax against amino acids 1 to 20 was purchased from Upstate Biotechnology. The MAb against cytochrome *c* oxidase subunit 4 (COX IV) was purchased from Molecular Probes. Isotype control Abs used were the ID4.5 [mouse immunoglobulin G<sub>2a</sub> (IgG<sub>2a</sub>)] MAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, Australia), the 107.3 mouse IgG<sub>1</sub> MAb purchased from PharMingen, and rabbit IgG from Sigma Chemical Co.

**Fresh melanoma isolates.** Isolation of melanoma cells from fresh surgical specimens was carried out as described previously (17).

**Apoptosis.** Quantitation of apoptotic cells by measurement of sub-G<sub>1</sub> DNA content using the propidium iodide (PI) method or by Annexin-V staining was carried out as described elsewhere (16, 18). Detection of apoptosis by DNA laddering assays was done as described previously (19). Briefly, the cell lysates were extracted with phenol/chloroform, and the DNA in the aqueous phase was precipitated with ethanol and resuspended in Tris-EDTA buffer. After digestion with 5 µg/mL RNase A for 1 h, DNA was reprecipitated, and DNA samples were electrophoretically separated on 1.5% agarose gel containing ethidium bromide (0.5 µg/mL). DNA was visualized by the Bio-Rad VersaDoc image system (Bio-Rad), and the gels were photographed.

**Flow cytometry.** Immunostaining on intact and permeabilized cells was carried out as described previously (16, 18). Analysis was carried out using a Becton Dickinson FACScan flow cytometer.

**Mitochondrial membrane potential ( $\Delta\Psi_m$ ).**  $\Delta\Psi_m$  was quantitated with JC-1 dye staining in flow cytometry as described previously (16).

**Western blot analysis.** Western blot analysis was carried out as described previously (16, 18). Labeled bands were detected by ImmunoStar HRP Chemiluminescent Kit, and images were captured, and the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (Bio-Rad).

**Preparation of mitochondrial and cytosolic fractions.** Methods used for subcellular fraction were similar to the methods described previously (18).

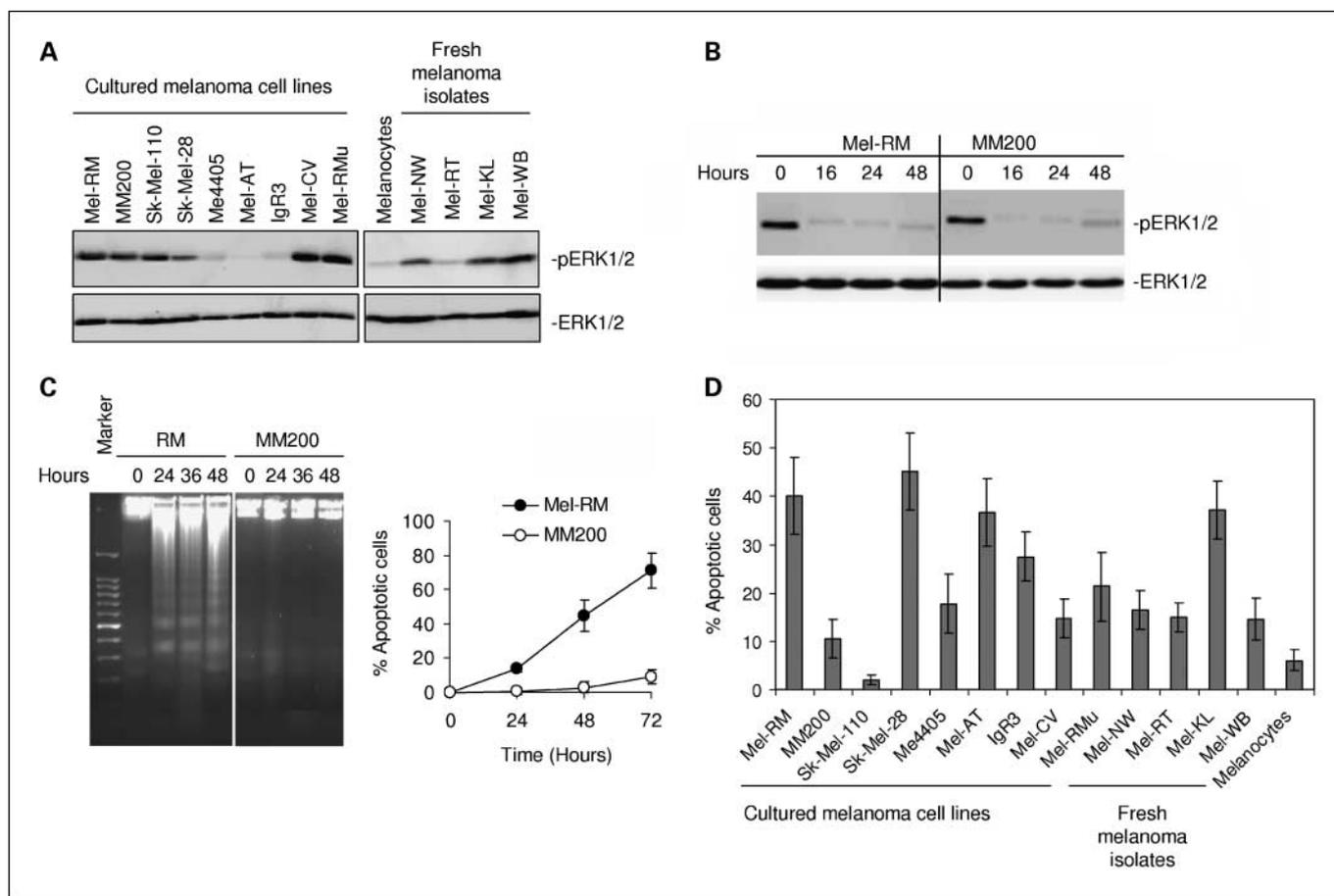
**Plasmid vector and transfection.** Stable melanoma transfectants of Bcl-2 were established by electroporation of the PEF-puro vector carrying human Bcl-2 provided by Dr. David Vaux (Walter and Eliza Hall Institute) and described elsewhere (18).

**Small RNA interference.** Melanoma cells were seeded at  $1 \times 10^5$  cells per well in 24-well plates and allowed to reach ~50% confluence on the day of transfection. The small interfering RNA (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon), the siGENOME SMARTpool MEK1 (M-003571-01-0010), the siGENOME SMARTpool AIF (M-011912-00-0010), the siGENOME SMARTpool Bim (M-004383-01-0010), the siGENOME SMARTpool PUMA (M-004380-01-0010), the siGENOME SMARTpool Bad (M-003870-02-0010), and the siGENOME SMARTpool Mcl-1 (M-004501-04-0010). The nontargeting siRNA control, SiConTRol-Non-targeting SiRNA pool (D-001206-13-20) was also obtained from Dharmacon. Cells were transfected with 50 to 100 nmol/L siRNA in Opti-MEM medium (Invitrogen) using LipofectAMINE reagent (Invitrogen) according to the manufacturer's transfection protocol. Twenty-four hours after transfection, the cells were treated with U0126 at 20 µmol/L for another 48 h before quantitation of apoptotic cells by measurement of sub-G<sub>1</sub> DNA content using the propidium iodide (PI) method in flow cytometry. Efficiency of siRNA was measured by Western blot analysis.

## Results

**Inhibition of MEK induces apoptosis in melanoma cells.** The constitutive activation status of ERK1/2 in a panel of melanoma cell lines and fresh melanoma isolates ( $\leq 3$  passages) and melanocytes is shown Fig. 1A. Among the cell lines, MM200, Sk-Mel-28, and Mel-RMu harbor the active BRAF mutation V600E (<sup>V600E</sup>BRAF), whereas the others carry the wild-type BRAF. All the cultured lines have wild-type N-Ras. As can be seen, the levels of ERK1/2 activation were not constantly associated with the <sup>V600E</sup>BRAF mutation. For example, Mel-RM and Mel-CV that contain the wild-type BRAF had high levels of ERK1/2 activation, whereas Sk-Mel-28 that harbors the <sup>V600E</sup>BRAF mutation exhibited only moderate levels of ERK1/2 activation. The levels of ERK1/2 activation also varied widely among the fresh melanoma isolates, although their BRAF mutational status has not been established. Normal melanocytes had little, if any, ERK1/2 activation, but the levels of ERK1/2 expression were comparable to those in melanoma cells.

Figure 1B and C shows that whereas treatment with the MEK inhibitor U0126 at 20 µmol/L inhibited ERK1/2 activation in both Mel-RM and MM200 cells, it only induced marked apoptosis in Mel-RM cells that could be detected as early as 24 h, with 43% and 72% of the cells being apoptotic at 48 and 72 h, respectively. MM200 cells seemed to be resistant to U0126-induced apoptosis with only 10% of the cells being apoptotic even at 72 h after treatment. Studies on a panel of melanoma cell lines revealed that there was a wide variation in sensitivity of melanoma cells to U0126-induced apoptosis, which was not associated with the <sup>V600E</sup>BRAF mutation (Fig. 1D). For instance, Mel-RM and Mel-AT that carried the wild-type BRAF were highly sensitive to U0126-induced apoptosis, whereas MM200 and Mel-RMu that harbored the <sup>V600E</sup>BRAF mutation had only low or moderate sensitivity to U0126-induced apoptosis. Similarly, the sensitivity to



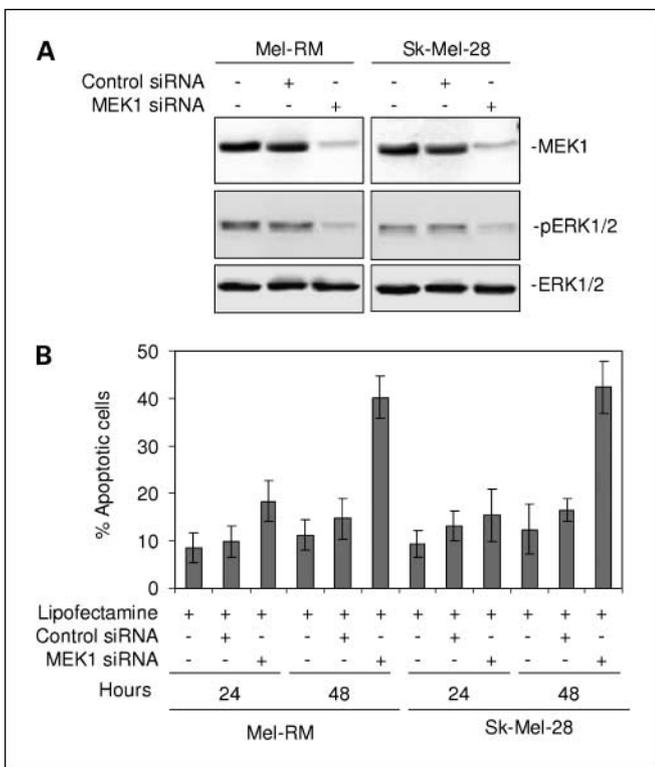
**Fig. 1.** Inhibition of MEK by U0126 induces apoptosis of melanoma cells. *A*, constitutive activation of ERK1/2 in cultured melanoma cells, fresh melanoma isolates, and melanocytes. Whole cell lysates from cells were subjected to Western blot analysis using an Ab against pERK1/2. The blots were subsequently reprobred with an antibody against ERK1/2. The data shown are representative of three individual experiments. *B*, U0126 inhibits activation/phosphorylation of ERK1/2 in melanoma cells. Whole cell lysates from Mel-RM and MM200 cells with or without treatment with U0126 (20  $\mu\text{mol/L}$ ) in DMEM for indicated time periods were subjected to Western blot analysis using an Ab against pERK1/2. The blots were subsequently reprobred with an antibody against ERK1/2. The data shown are representative of three individual experiments. *C*, U0126 induces apoptosis of melanoma cells. Mel-RM and MM200 cells treated with U0126 (20  $\mu\text{mol/L}$ ) for indicated intervals were subjected to either DNA laddering assays (*left*) or measurement of sub-G<sub>1</sub> content by the propidium iodide method using flow cytometry (*right*). The data shown are either representative of three individual experiments (*left*) or the mean  $\pm$  SE of three individual experiments (*right*). *D*, induction of apoptosis by U0126 in a panel of melanoma cell lines, fresh melanoma isolates, and melanocytes. Cells treated with U0126 (20  $\mu\text{mol/L}$ ) for 48 h were subjected to measurement of sub-G<sub>1</sub> content by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE.

U0126-induced apoptosis varied between fresh melanoma isolates with Mel-KL being sensitive, while the others (Mel-NW, Mel-RT, and Mel-WB) being relatively resistant. Inhibition of MEK by U0126 had minimal toxicity toward normal melanocytes (Fig. 1D).

To confirm that U0126-induced apoptosis of melanoma was due to inhibition of MEK/ERK signaling, we silenced MEK1 expression in two cultured melanoma cell lines that were relatively sensitive to apoptosis induced by U0126, Mel-RM, and Sk-Mel-28 (Fig. 1D), by transfecting the cells with MEK1 siRNA. As shown in Fig. 2A, siRNA knockdown of MEK1 inhibited its expression by 85% and 90% in Mel-RM and Sk-Mel-28 cells, respectively. Consequently, the levels of ERK1/2 activation were substantially blocked in both cell lines. Figure 2B shows that inhibition of MEK1 by siRNA resulted in marked apoptotic cell death in both cell lines by 48 h after transfection.

**Apoptosis of melanoma induced by inhibition of MEK involves activation of Bax and Bak and changes in mitochondria.** The activation status of Bax and Bak in permeabilized Mel-RM

and MM200 cells before and after treatment with U0126 was studied by using antibodies that specifically recognize activated Bax and Bak, respectively, in flow cytometry (20). Figure 3A shows that U0126 induced marked activation of Bax and Bak in Mel-RM cells that was observed as early as 16 h. By 36 h, the mean fluorescence intensities (MFI) for Bax and Bak were increased by 4 and 3.5 times, respectively. In contrast, exposure to U0126 resulted in only minimal activation of Bax and Bak in MM200 cells. Similarly, U0126 induced marked reduction in  $\Delta\Psi_m$  in Mel-RM but not in MM200 cells (Fig. 3B). These changes were associated with release of cytochrome *c*, Smac/DIABLO, and AIF from mitochondria into the cytosol, which could be observed as early as 16 h, and increased markedly by 36 h after treatment in Mel-RM cells (Fig. 3C). In contrast, release of these proteins from the mitochondria was much less pronounced in MM200 cells even at 72 h (Fig. 3C and data not shown). The role of mitochondria in U0126-induced apoptosis of melanoma was further confirmed with Mel-RM cells transfected with cDNA encoding Bcl-2. As shown in Fig. 3D, the levels of U0126-induced



**Fig. 2.** Knockdown of MEK1 by siRNA induces apoptosis of melanoma cells. **A**, siRNA knockdown of MEK1 inhibits activation/phosphorylation of ERK. Mel-RM and Sk-Mel-28 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of MEK1, pERK1/2, and ERK1/2 expression. The data shown are representative of three individual experiments. **B**, MEK1 siRNA induces apoptosis of melanoma cells. Mel-RM and Sk-Mel-28 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, the cells were switched into normal culture medium for indicated time periods followed by measurement of sub-G<sub>1</sub> content by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE.

apoptosis in Bcl-2-transfected Mel-RM cells were markedly decreased in comparison with those in vector-alone-transfected cells.

**Apoptosis of melanoma cells induced by inhibition of MEK is dependent on the release of AIF but not activation of caspases.** Inhibition of MEK by U0126 induced the activation of caspase-9 and caspase-3 in Mel-RM cells as evidenced by the appearance of the p37 form of cleaved caspase-9 and the p17 form of activated caspase-3, respectively (Fig. 4A). In contrast, no appreciable activation of caspase-8 could be detected within 36 h. At 48 h after treatment with U0126, the levels of proenzymes of caspase-9, caspase-3, and caspase-8 were markedly reduced. Little or no activation of the caspases could be detected in MM200 cells after treatment with U0126. Notably, PARP was expressed at barely detectable levels in this cell line. The difference in activation of caspase-3 between Mel-RM and MM200 cells was confirmed by using a MAb that specifically recognizes the activated form of caspase-3 in flow cytometry (Fig. 4B). Despite this evidence for activation of caspases, a pan-caspase inhibitor (z-VAD-fmk) did not inhibit U0126-induced apoptosis or changes in Bax, Bak, and mitochondria, whereas it blocked apoptosis induced by the TRAIL that was used as a control (Fig. 4C and data not shown).

We next examined the role of AIF in U0126-induced apoptosis by using siRNA knockdown of AIF in two melanoma cell lines, Mel-RM and Sk-Mel-28, which were relatively sensitive to apoptosis induced by the inhibition of MEK/ERK (Fig. 1D). As shown in Fig. 4D, siRNA knockdown of AIF inhibited AIF expression by 92% in Mel-RM and 85% in Sk-Mel-28 cells. The levels of apoptosis induced by U0126 in cells transfected with AIF siRNA were markedly reduced in comparison with cells transfected with the control siRNA in both Mel-RM and Sk-Mel-28 cells ( $P < 0.01$ , two-tailed Student's *t* test).

**Inhibition of MEK up-regulates the BH3-only proteins, Bim, PUMA, and Bad, but down-regulates the antiapoptotic protein Mcl-1.** As shown in Fig. 5A, treatment with U0126 resulted in a marked increase in the levels of Bim<sub>EL</sub> in both Mel-RM and MM200 cells that could be detected as soon as 3 h. An increase in the levels of Bim<sub>L</sub> and Bim<sub>S</sub> was also evident in Mel-RM cells by 24 h. The antibody against Bim detected an extra band with reduced electrophoretic motility, which corresponds to phosphorylated Bim<sub>EL</sub> (21, 22), and was rapidly decreased after treatment with U0126 in Mel-RM cells but not in MM200 cells.

Treatment with U0126 up-regulated PUMA in both Mel-RM and MM200 cells with different kinetics. PUMA in Mel-RM cells increased within 3 h and peaked by 6 h, whereas PUMA in MM200 cells peaked at 3 h followed by a rapid decrease. Similarly, there was rapid up-regulation of Bad in Mel-RM cells. Bad was expressed at high levels in MM200 cells even before treatment and was not changed by exposure to U0126. Noxa was constitutively expressed at low levels in Mel-RM, but not detectable in MM200 cells. The levels of Noxa were decreased in Mel-RM cells by 24 h after treatment with U0126. Inhibition of MEK by U0126 did not regulate the levels of expression of the proapoptotic proteins Bid, Bax, and Bak in both Mel-RM and MM200 cells.

Figure 5A also shows that treatment with U0126 resulted in a rapid but transient increase in the expression levels of the antiapoptotic Bcl-2 family member, Mcl-1, in Mel-RM cells, which peaked at 6 h followed by a marked decrease. There was no appreciable alteration in the expression levels of Mcl-1 in MM200 cells within 6 h, but by 24 h, the levels of Mcl-1 seemed to be reduced. Notably, Mcl-1 was expressed at lower levels in Mel-RM than MM200 cells before exposure to U0126. The levels of expression of another two prosurvival Bcl-2 family members Bcl-2 and Bcl-X<sub>L</sub> remained unaltered in both Mel-RM and MM200 cells after treatment with U0126. Figure 5B shows that knockdown of MEK1 with siRNA induced similar changes in the expression of Bim, PUMA, and Mcl-1 to those induced by U0126 in Mel-RM and MM200 cells.

We also studied the effects of U0126 on the expression of Bim, PUMA, and Mcl-1 on one sensitive and one resistant fresh melanoma isolates, Mel-KL and Mel-WB, and normal melanocytes. As shown in Fig. 5C, the similar patterns of changes in Bim<sub>EL</sub>, PUMA, and Mcl-1 induced by U0126 were observed in Mel-KL and Mel-WB to those seen in Mel-RM and MM200, respectively. Bim<sub>L</sub> and Bim<sub>S</sub> were not detected in either Mel-KL or Mel-WB even after treatment. Neither Bim (Bim<sub>EL</sub>, Bim<sub>L</sub>, and Bim<sub>S</sub>) nor PUMA could be reliably detected in normal melanocytes before and after exposure to U0126. Mcl-1 was expressed at low levels that were not changed by treatment with U0126 in melanocytes.

**PUMA, Bim, and Mcl-1, but not Bad, play critical roles in regulating the sensitivity of melanoma cells to apoptosis induced by inhibition of MEK.** The role of Bim and PUMA in U0126-induced apoptosis was tested by knockdown with specific siRNA constructs for Bim and PUMA in Mel-RM and Sk-Mel-28 cells, respectively. As shown in Fig. 6A and B, siRNA knockdown of Bim markedly inhibited the expression of Bim<sub>EL</sub>, Bim<sub>L</sub>, and Bim<sub>S</sub> and blocked U0126-induced apoptosis by 55% in Mel-RM and 52% in Sk-Mel-28 cells. Similarly, siRNA knockdown of PUMA reduced the levels of U0126-induced apoptosis by 54% in Mel-RM and 57% in Sk-Mel-28 cells (Fig. 6A and B). Consistent with this, siRNA knockdown of Bim or PUMA markedly inhibited U0126-induced activation of Bax and Bak and reduction in  $\Delta\Psi_m$  in both Mel-RM and Sk-Mel-28 cells (data not shown).

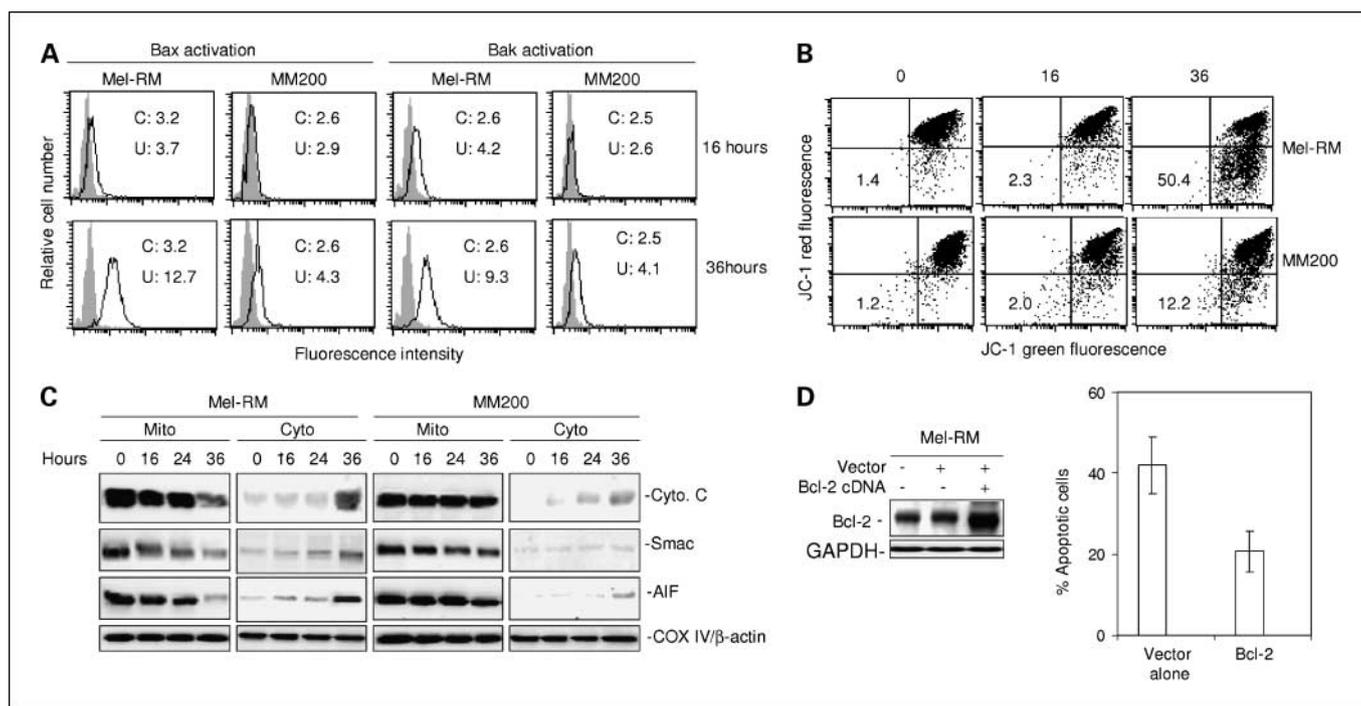
To ascertain whether activation of Bad is sufficient to account for the induction of apoptosis of melanoma cells by inhibition of MEK as reported by others (15), we transfected Mel-RM and Sk-Mel-28 cells with a Bad-specific siRNA construct. As shown in Fig. 6A, siRNA knockdown of Bad markedly inhibited the expression of Bad, but had little or no effect on the levels of apoptosis induced by U0126 in both cell lines (Fig. 6B).

The role of Mcl-1 in U0126-induced apoptosis of melanoma was also tested by transfecting a Mcl-1-specific siRNA construct

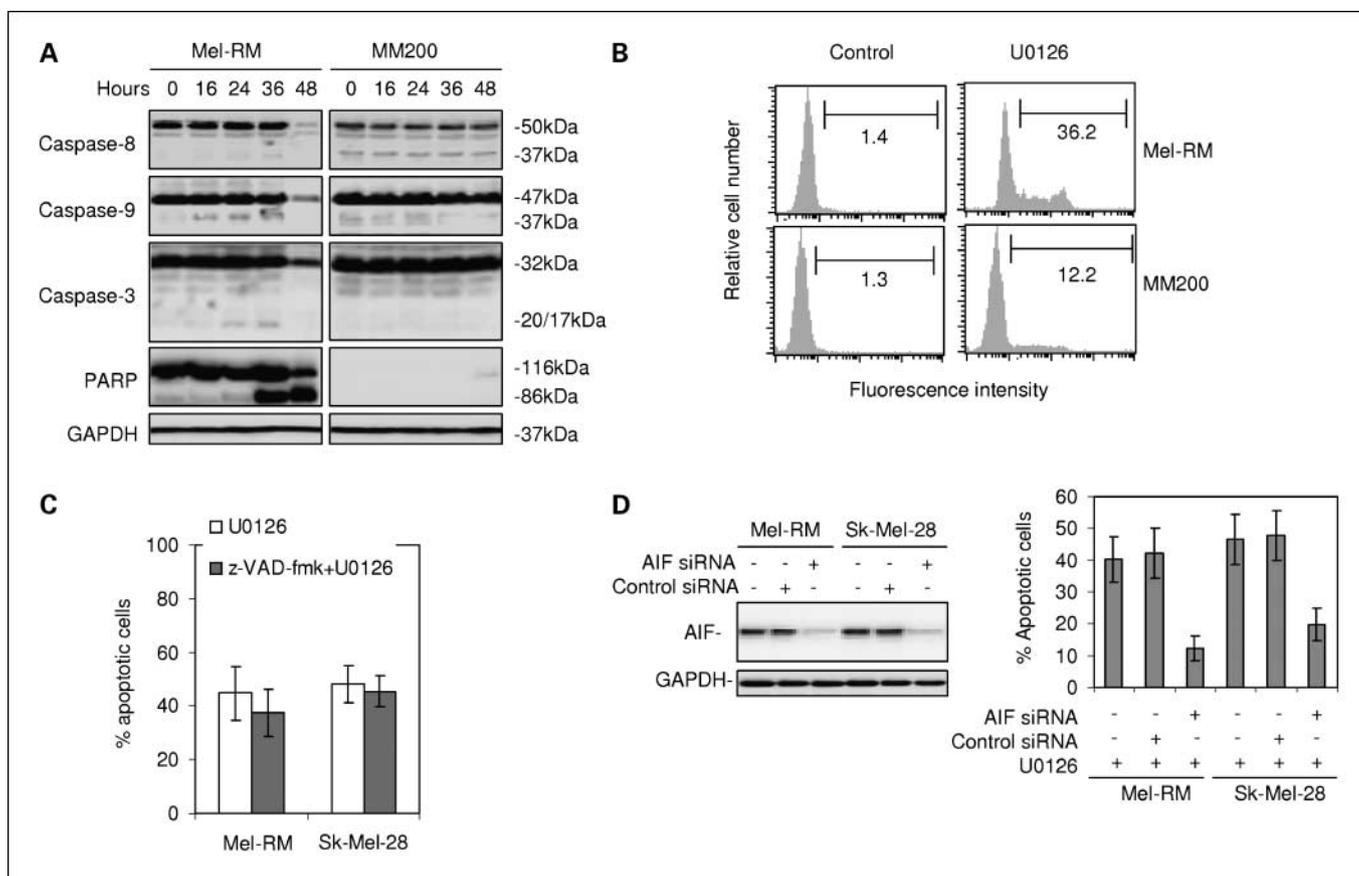
into Mel-RM and MM200 cells. The latter were known to be resistant to apoptosis induced by the inhibition of MEK. As shown in Fig. 6C, siRNA knockdown of Mcl-1 inhibited Mcl-1 expression by more than 90% in both cell lines. Notably, inhibition of Mcl-1 by siRNA caused apoptosis in both Mel-RM and MM200 cells (Fig. 6D). Although the Mcl-1 siRNA had only a moderate effect on the levels of U0126-induced apoptosis in Mel-RM cells, it markedly sensitized MM200 cells to U0126-induced apoptosis (Fig. 6D). Similar results were observed when Mcl-1 was knocked down by siRNA in the relatively resistant fresh melanoma isolate Mel-WB (Fig. 6C and D).

## Discussion

Several MEK inhibitors have reached the stage of clinical evaluation in melanoma. These include PD0325901 made by Pfizer and AZD6244 made by Astra Zeneca. Their role in inhibition of cell division is well studied, but their ability to induce apoptosis is less understood (4, 23). In the present study, we have used a selective inhibitor of MEK (U0126) to better characterize their induction of apoptosis in melanoma. Our results showed firstly that induction of apoptosis by a MEK inhibitor (U0126) required prolonged culture in



**Fig. 3.** U0126 induces mitochondrial apoptotic events. **A**, U0126 induces activation of Bax and Bak. Mel-RM and MM200 cells treated with U0126 (20  $\mu\text{mol/L}$ ) for the indicated intervals were subjected to measurement of activation of Bax and Bak by flow cytometry using antibodies that specifically recognize activated Bax and Bak, respectively. The filled and open histograms were generated from control untreated (C) and U0126-treated cells (U), respectively. The numbers represent MFIs. The data shown are representative of three individual experiments. **B**, U0126 induces reduction in  $\Delta\Psi_m$ . Mel-RM and MM200 cells treated with U0126 (20  $\mu\text{mol/L}$ ) for the indicated intervals were subjected to measurement of  $\Delta\Psi_m$  by JC-1 staining in flow cytometry. The number in each left bottom quadrant represents the percentage of cells with reduction in  $\Delta\Psi_m$ . The data shown are representative of three individual experiments. **C**, U0126 induces mitochondrial release of cytochrome c, Smac/DIABLO, and AIF. Mitochondrial and cytosolic fractions from Mel-RM and MM200 cells treated with U0126 (20  $\mu\text{mol/L}$ ) for the indicated intervals were subjected to Western blot analysis. Western blot analysis of COX IV or  $\beta$ -actin levels was included to show relative purity of the mitochondrial or cytosolic fractions. The data shown are representative of three individual experiments. **D**, overexpression of Bcl-2 inhibits U0126-induced apoptosis. Left, Bcl-2 was overexpressed in Mel-RM and Me4405 cells transfected with cDNA encoding Bcl-2, but not in the cells transfected with the vector alone. Whole cell lysates were subjected to Western blot analysis. The data shown are representative of two individual experiments. Right, Mel-RM and Me4405 cells transfected with c-DNA for Bcl-2 or vector alone were treated with U0126 (20  $\mu\text{mol/L}$ ) 48 h before measurement of apoptosis by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE.



**Fig. 4.** AIF, but not the caspase cascade, plays a crucial role in U0126-induced apoptosis of melanoma. **A**, U0126 induces caspase activation. Whole cell lysates from Mel-RM and MM200 cells treated with U0126 (20  $\mu$ mol/L) for the indicated time periods were subjected to Western blot analysis. The data shown are representative of three individual experiments. **B**, Mel-RM and MM200 cells with or without treatment with U0126 (20  $\mu$ mol/L) for 36 h were subjected to flow cytometry analysis of caspase-3 activation using an antibody that specifically recognizes the activated form of caspase-3. The data shown are representative of three individual experiments. **C**, the pan-caspase inhibitor z-VAD-fmk does not block U0126-induced apoptosis of melanoma cells. Mel-RM and Sk-Mel-28 cells with or without pretreatment with z-VAD-fmk (20  $\mu$ mol/L) for 1 h were treated with U0126 (20  $\mu$ mol/L) for another 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. **D**, knockdown of AIF by siRNA inhibits U0126-induced apoptosis. Left, expression of AIF was measured by Western blot analysis of whole cell lysates from Mel-RM and Sk-Mel-28 cells 24 h after transfection with the control or AIF siRNA. The data shown are representative of three individual experiments. Right, Mel-RM and Sk-Mel-28 cells were transfected with the control or AIF siRNA. Twenty-four hours later, the cells were exposed to U0126 (20  $\mu$ mol/L) for a further 48 h. Apoptosis was then measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE.

the inhibitor. Secondly, there was marked variability in the levels of apoptosis induced by inhibition of MEK in melanoma cell lines and fresh melanoma isolates, but this variability did not seem related to the presence of mutated BRAF in the melanoma cell lines. This result contrasted with a previous study where MEK inhibition of cyclin D1 expression in 24-h assays was found to be more marked in melanoma cells with BRAF mutations (23). The difference may reflect the different assays and durations of exposure to the MEK inhibitors. A potential impact of activating mutations of N-Ras on the current results in cultured cell lines could be ruled out in that all the lines used in this study carried wild-type N-Ras.

Apoptosis induced by the MEK inhibitor was unusual in that although it involved activation of Bax and Bak, changes in mitochondria, and activation of caspases, it proved to be independent of the caspase cascade. Instead, apoptosis was dependent on AIF, which has the ability to translocate from mitochondria to the nucleus, inducing DNA fragmentation independently of caspase activity (24–26). This result was similar to our previous studies on apoptosis induced by

the general protein kinases inhibitor staurosporine, which induced a delayed apoptotic response caused by AIF (25). Moreover, AIF was also reported to be the mediator of apoptosis of melanoma cells induced by the RAF inhibitor sorafenib despite activation of caspases (24). It remains unclear why AIF, but not the caspase cascade, seems to be the mediator of apoptosis induced by the signaling pathway inhibitors. This was not due to IAP-mediated inhibition of caspases as the caspase-3 substrate PARP was proteolytically cleaved (15, 23). It is also unknown if AIF induces the apoptosis of melanoma cells by directly binding to and cleaving DNA and/or other cofactors such as endonuclease G is also involved (26, 27). Activation of the caspase cascade by other MEK inhibitors, including PD98059 and CI-1040, in melanoma cells was also reported, but the role of AIF in induction of apoptosis in these studies was not known.

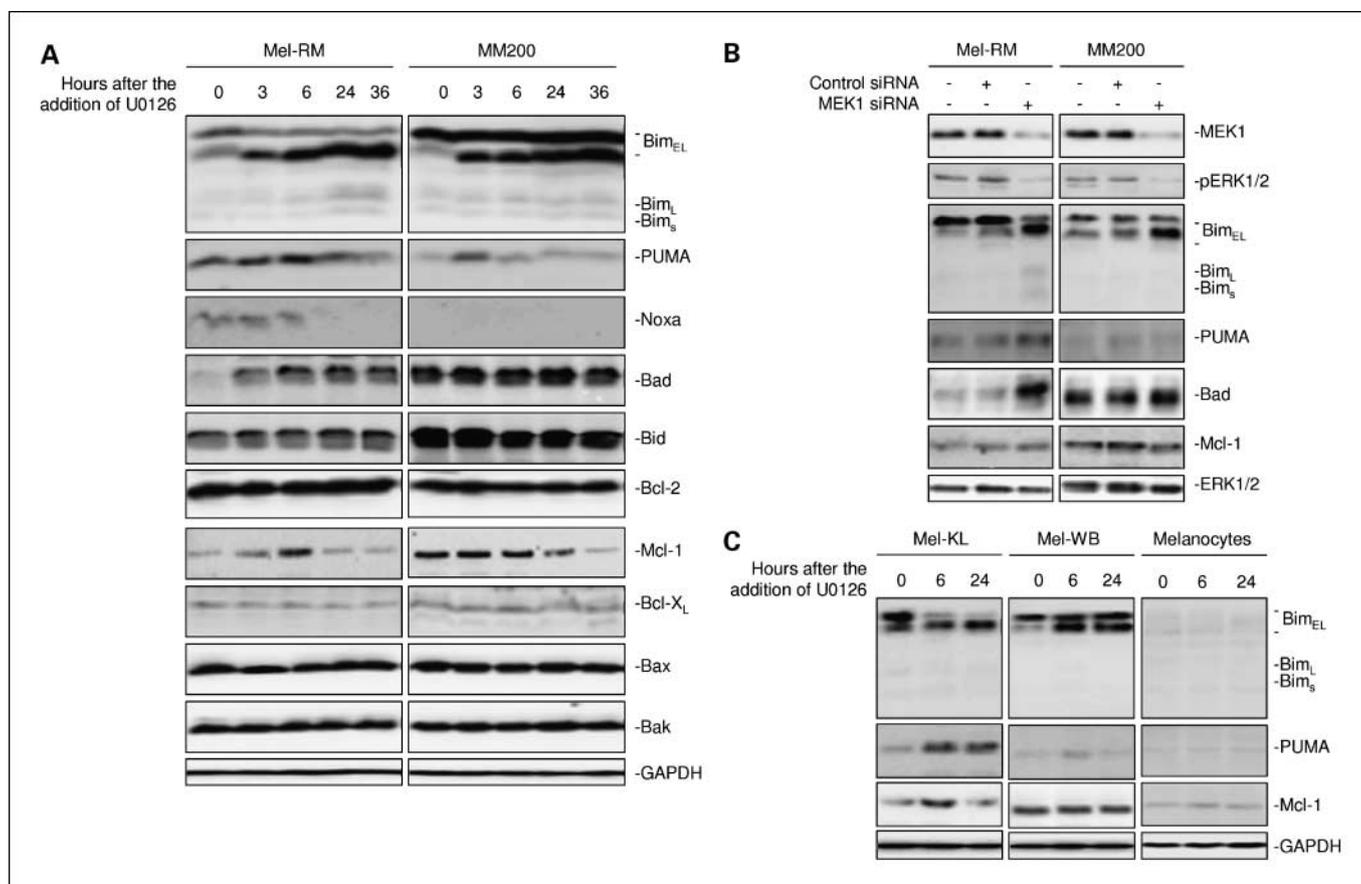
To better understand the mechanism that regulates the sensitivity of melanoma cells to apoptosis induced by inhibition of MEK, we compared changes in Bcl-2 family proteins between sensitive (Mel-RM) and resistant (MM200) melanoma cells. Inhibition of MEK by either U0126 or MEK1

siRNA up-regulated PUMA in both cell lines, but the levels were higher and persisted longer in the apoptosis-sensitive Mel-RM cells. Inhibition of MEK also caused a rapid increase in Bim<sub>EL</sub> expression in both Mel-RM and MM200 cells, but up-regulation of Bim<sub>L</sub> and Bim<sub>S</sub> was only observed in Mel-RM cells. Bim<sub>L</sub> and Bim<sub>S</sub> are known to be more potent in inducing apoptosis (28). These results are consistent with the idea that the increased levels of PUMA and Bim may have initiated apoptosis through the activation of Bax and Bak (11–13, 28). The decreased phosphorylation of Bim<sub>EL</sub> in the sensitive Mel-RM cells may have contributed to the apoptosis signaling (29). PUMA and Bim are able to potently bind to all the antiapoptotic proteins of the Bcl-2 family (30). The importance of PUMA and Bim in apoptosis induced by inhibition of MEK was confirmed by siRNA knockdown, which inhibited U0126-induced apoptosis in the apoptosis-sensitive cells. Our current results did not support a major role of activation of Bad by the inhibition of MEK in the induction of apoptosis of melanoma cells (15) in that inhibition of Bad by siRNA did not block apoptosis induced by U0126.

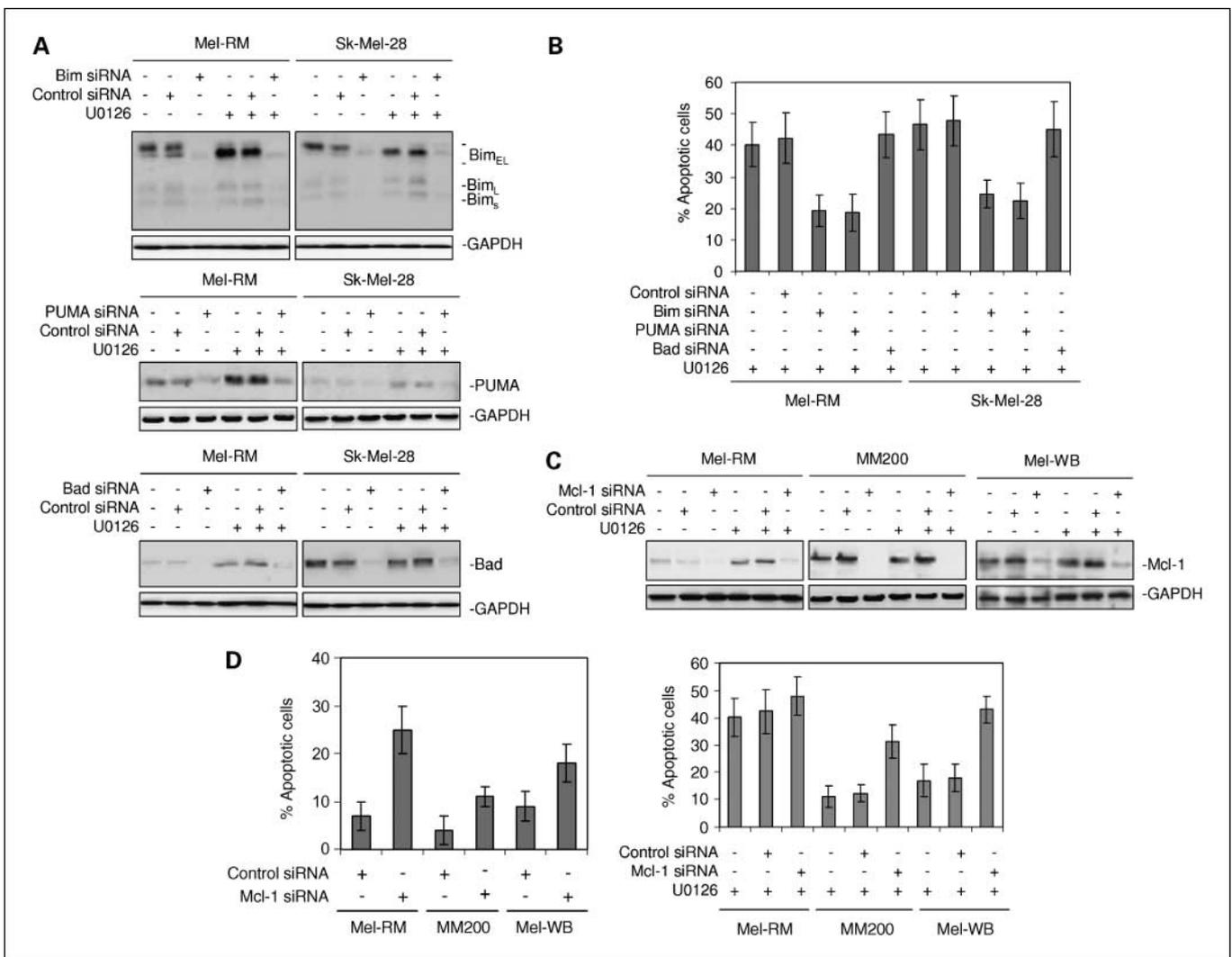
In addition to changes in PUMA and Bim, there were substantial differences in the concentrations of the antiapoptotic Mcl-1 protein with a transient increase being seen in the

sensitive line (Mel-RM), whereas higher levels were sustained up to 24 h in the resistant line (MM200). There was no significant difference in the concentrations of Bcl-2 and Bcl-X<sub>L</sub> between the Mel-RM and MM200 cells. Mcl-1 was shown to play a unique apical role in apoptosis as elimination of Mcl-1 is required at an early stage for induction of apoptosis (13). Consistent with this, inhibition of Mcl-1 by siRNA resulted in low to moderate levels of apoptosis in both the sensitive and resistant melanoma cells, further emphasizing the important role of Mcl-1 in protecting melanoma cells from apoptosis. We have shown elsewhere that Mcl-1 protein levels increase during progression of melanoma (31). Should these studies on a sensitive and resistant line apply in general, our results strongly suggest that MEK/ERK-mediated survival of melanoma cells is controlled, at least in part, by the Bcl-2 family members PUMA, Bim, and Mcl-1.

A paradoxical finding of this study is that the levels of the Mcl-1 protein in the apoptosis-sensitive Mel-RM cells experienced a transient but substantial increase. We have found by real-time PCR analysis that the mRNA levels of Mcl-1 were not up-regulated by U0126 (data not shown). A possible explanation for this is that binding of Mcl-1 with its proapoptotic partners such as PUMA and Bim during initiation of apoptosis



**Fig. 5.** Inhibition of MEK regulates the expression of Bim, PUMA, and Mcl-1. *A*, U0126 alters the expression levels of Bim, PUMA, and Mcl-1 in melanoma cell lines. Whole cell lysates from Mel-RM and MM200 cells treated with U0126 (20 μmol/L) for indicated time periods were subjected to Western blot analysis. The data shown are representative of three individual experiments. *B*, effects of MEK1 siRNA on expression of Bim, PUMA, and Mcl-1. Mel-RM and MM200 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, whole cell lysates were subjected to Western blot analysis of MEK1, pERK1/2, Bim, PUMA, Mcl-1, and ERK1/2 expression. The data shown are representative of three individual experiments. *C*, U0126 alters the expression levels of Bim, PUMA, and Mcl-1 in fresh melanoma isolates. Whole cell lysates from Mel-KL and Mel-WB cells treated with U0126 (20 μmol/L) for indicated time periods were subjected to Western blot analysis. The data shown are representative of two individual experiments.



**Fig. 6.** Bim, PUMA, and Mcl-1 play important roles in regulating the apoptosis of melanoma cells induced by the inhibition of MEK. **A**, knockdown of Bim, PUMA, and Bad by siRNA decreases the levels of expression of Bim, PUMA, and Bad, respectively, in the absence or presence of U0126. Mel-RM and Sk-Mel-28 cells were transfected with the control, Bim, PUMA, or Bad siRNA. Twenty-four hours later, the cells were treated with U0126 (20  $\mu\text{mol/L}$ ) for a further 24 h. Whole cell lysates were then subjected to Western blot analysis. **B**, inhibition of Bim or PUMA by siRNA blocks U0126-induced apoptosis. Mel-RM and Sk-Mel-28 cells were transfected with the control, Bim, PUMA, or Bad siRNA. Twenty-four hours later, the cells were exposed to U0126 (20  $\mu\text{mol/L}$ ) for a further 48 h. Apoptosis was then measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. **C**, knockdown of Mcl-1 by siRNA decreases the levels of Mcl-1 expression. Mel-RM and MM200 cells and the fresh melanoma isolate Mel-WB cells were transfected with the control or Mcl-1 siRNA. Twenty-four hours later, the cells were treated with U0126 (20  $\mu\text{mol/L}$ ) for a further 24 h. Whole cell lysates were then subjected to Western blot analysis. **D**, inhibition of Mcl-1 by siRNA induces apoptosis of melanoma (left) and sensitizes melanoma cells to U0126-induced apoptosis (right). Left, Mel-RM and MM200 cells and the fresh melanoma isolate Mel-WB cells were transfected with the control or Mcl-1 siRNA. Twenty-four hours later, the cells were switched into normal culture medium for a further 48 h followed by measurement of sub-G<sub>1</sub> content by the propidium iodide method using flow cytometry. The data shown are the mean  $\pm$  SE of three individual experiments. Right, 24 h after transfection, the cells were treated with U0126 (20  $\mu\text{mol/L}$ ) for 48 h followed by measurement of sub-G<sub>1</sub> content by the propidium iodide method using flow cytometry. The data (y-axis) are expressed as percentages of apoptotic cells with those that resulted from the transfection of the control or Mcl-1 siRNA alone being subtracted. Columns, mean of three individual experiments; bars, SE.

may, as described by others (32), increase its stability, thus delaying its degradation.

The basis for the differences in Bcl-2 family proteins between melanoma cells that are sensitive or resistant to inhibition of MEK is largely unknown. They do not seem related to the levels of activation of ERK1/2 or the presence of mutations in BRAF. Regulation of Bcl-2 family proteins by other survival signaling pathways such as the Akt/PKB and nuclear factor- $\kappa$ B pathways may also be involved (33, 34). However, these studies have shown that apoptosis induced by inhibition of MEK in cultured melanoma cells is caspase independent and is mediated by the up-regulation/activation of the BH3-only proteins PUMA and

Bim and down-regulation of the antiapoptotic Bcl-2 family member Mcl-1.

The relevance of these findings to treatment of melanoma patients with MEK inhibitors was supported by our finding that the inhibition of MEK by U0126 also induced apoptosis in fresh melanoma isolates, and that sensitivity to apoptosis could be enhanced by the inhibition of Mcl-1. This may reflect more closely the *in vivo* status of the susceptibility of melanoma cells to apoptosis induced by MEK inhibitors. Taken together, our results support the clinical use of selective MEK inhibitors in the treatment of melanoma and suggest that cotargeting Mcl-1 and the MEK/ERK pathway requires further investigation.

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