

# Camptothecin Induces Apoptosis in Cancer Cells via MicroRNA-125b-Mediated Mitochondrial Pathways<sup>§</sup>

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## ABSTRACT

Camptothecin (CPT) is an effective chemotherapeutic agent for treatment of patients with cancer. The mechanisms underlying CPT-mediated responses in cancer cells are not fully understood. MicroRNA (miRNA) play important roles in tumorigenesis and drug sensitivity. However, the interaction between camptothecin and miRNA has not been previously explored. In this study, we verified that miR-125b was down-regulated in CPT-induced apoptosis in cancer cells and that ectopic expression of miR-125b partially restored cell viability and inhibited cell apoptosis that was induced by CPT. In addition, we demonstrated that CPT induced apoptosis in cancer cells by miR-125b-mediated mitochondrial pathways via targeting to the 3'-untranslated (UTR) regions of Bak1, Mcl1, and p53. A significant increase in Bak1, Mcl1, and p53 protein levels was

detected in response to the treatments of CPT. It is noteworthy that the expression levels of Bak1, Mcl1, and p53 increased in a time-dependent manner and negatively correlated with miR-125b expression. It is noteworthy that we revealed that miR-125b directly targeted the 3' UTR regions of multiple genes in a CPT-induced mitochondrial pathway. In addition, most targets of miR-125b were proapoptotic genes, whereas some of the targets were antiapoptotic genes. We hypothesized that miR-125b may mediate the activity of chemotherapeutic agents to induce apoptosis by regulating multiple targets. This is the first report to show that camptothecin induces cancer cell apoptosis via miRNA-mediated mitochondrial pathways. The results suggest that suppression of miR-125b may be a novel approach for the treatment of cancer.

## Introduction

Camptothecin (CPT) and its derivatives have recently emerged as important chemotherapeutic agents against various malignancies, including solid tumors and hematopoietic malignancies (Zunino and Pratesi, 2004). One of the major modes of action of these chemotherapeutic drugs may be via the activation of apoptosis (Makin and Dive, 2001). Indeed, several researchers have reported that CPT induces apoptosis in various cancer cells (Sánchez-Alcázar et al., 2000; Tan et al., 2009). However, the detailed molecular mechanisms are not fully understood. Understanding the mechanism of CPT is important for future clinical applications.

MicroRNAs (miRNAs), which are a new class of non-protein-coding genes, are thought to regulate the expression of up to 30% of human genes by inhibiting mRNA translation or inducing its degradation (Lewis et al., 2005). MiRNAs are now recognized to play key roles in cancer cell proliferation, differentiation, and apoptosis (Chen et al., 2004; Cheng et al., 2005; Karp and Ambros, 2005). For instance, miR-21 is an antiapoptotic factor (Li et al., 2009) and miR-34 is a downstream target of p53 and an inducer of cell death in various cancers (He et al., 2007). Several studies have suggested a role for miRNAs in modulating sensitive or resistant phenotypes to chemotherapeutics, calling for further investigation and validation of miRNA functions and targets to improve sensitivity to cancer treatments. MiR-125b, a homolog of lin-4, is the first miRNA discovered as an important regulator of developmental timing in *Caenorhabditis elegans* (Olsen and Ambros, 1999). Aberrant expression of miR-125b has been reported in multiple cancers such as leukemia, glioma, and prostate cancer (Bousquet et al., 2008; Xia et al., 2009; Shi et al., 2011) and has been recognized as an oncomir or a tumor suppressor (Scott et al., 2007; Shi et al., 2007). Recent

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**ABBREVIATIONS:** CPT, camptothecin; CPT, camptothecin; miRNA or miR, microRNA; qRT-PCR, quantitative real-time-polymerase chain reaction; siRNA, small interfering RNA; wt, wild type; UTR, untranslated region; MRE, miRNA response element; NC, negative control.

studies have reported multiple functions of miR-125b, including proliferation, differentiation, and apoptosis (Le et al., 2009b; Xia et al., 2009; Zhou et al., 2010). Several studies have revealed that in cells, miR-125b confers resistance to chemotherapeutic drugs such as all-*trans* retinoic acid and paclitaxel (Xia et al., 2009; Zhou et al., 2010), indicating that miR-125b may be a drug target or involved in chemotherapeutic agent-induced apoptosis. However, the mechanisms that underlie the involvement of miR-125b in CPT-induced apoptosis are not known.

In this study, we demonstrated that miR-125b was down-regulated in CPT-induced apoptosis in cancer cells and that ectopic expression of miR-125b partially restored cell viability and inhibited cell apoptosis induced by CPT. More importantly, we reported the identification of many genes that regulate mitochondrial pathways as the targets of miR-125b, suggesting that suppression of miR-125b may be a novel approach for the treatment of cancer.

## Materials and Methods

**Cell Culture and Reagents.** Human cervical cancer HeLa cells and human prostate cancer PC3 cells were cultured in Dulbecco's modified Eagle's medium, human immortalized myelogenous leukemia K562 cells, human T-cell lymphoblast-like CCRF-CEM cells, human non-small-cell lung carcinoma NCI-H1299 cells, human colon carcinoma HCT-15 cells, normal human bronchial epithelium HBE cells, and human cholangiocarcinoma RBE cells were cultured in RPMI 1640 medium, and human cervical carcinoma C-33A cells were cultured in Eagle's minimal essential medium. All types of media contained 10% heat-inactivated fetal bovine serum. The cells were incubated at 37°C in 5% CO<sub>2</sub>. Camptothecin was obtained from Sigma-Aldrich (St. Louis, MO). All other materials were from standard sources and of the highest purity that is available commercially.

**RNA Isolation and Quantitative RT-PCR.** Total RNA derived from cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Quantitative RT-PCR analysis was performed by using SYBR Green (Takara Bio, Kyoto, Japan) on an ABI StepOne real-time PCR machine (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. The expression level of mature miRNA was normalized to the U6 expression level. The PCR conditions were 40 cycles at 95°C for 15 s and then 60°C for 30 s. For quantitative real-time PCR of mRNAs,  $\beta$ -actin served as an internal control. Sequences of the primers and probes are listed in Supplemental Table 1.

**Cell Transfection.** The cells were transfected using the Neon Transfection System (Invitrogen) with 100 pmol of oligonucleotides in 10- $\mu$ l reactions. The hsa-miR-125b miRNA precursor (miR-125b), PremiR negative control (miR-NC), hsa-miR-125b miRNA inhibitor, and anti-miR negative control, were purchased from Ambion (Austin, TX). Various siRNAs were purchased from RiboBio (Guangzhou, China).

**Western Blotting.** Protein extracts were boiled in radioimmunoprecipitation assay buffer or 1% SDS. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene difluoride membranes (Millipore) by electrophoretic transfer and reacted with anti-MCL1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-BAK (Biogenex, San Ramon, CA), anti-GAPDH (Proteintech Group, Inc., Chicago, IL), or anti- $\beta$ -tubulin (Cell Signaling Technology, Danvers, MA). Immunoreactivity was determined using the enhanced chemiluminescence method (Millipore, Billerica, MA) according to the manufacturer's instructions.

**Apoptosis Assessment by Annexin V/Propidium Iodide Staining.** After cells were treated with or without CPT, the cells were centrifuged and resuspended in 500  $\mu$ l of staining solution (containing annexin V fluorescein and propidium iodide in HEPES

buffer) (annexin V:FITC apoptosis detection kit; BD Pharmingen, San Diego, CA). After incubation at room temperature for 15 min, cells were analyzed using flow cytometry.

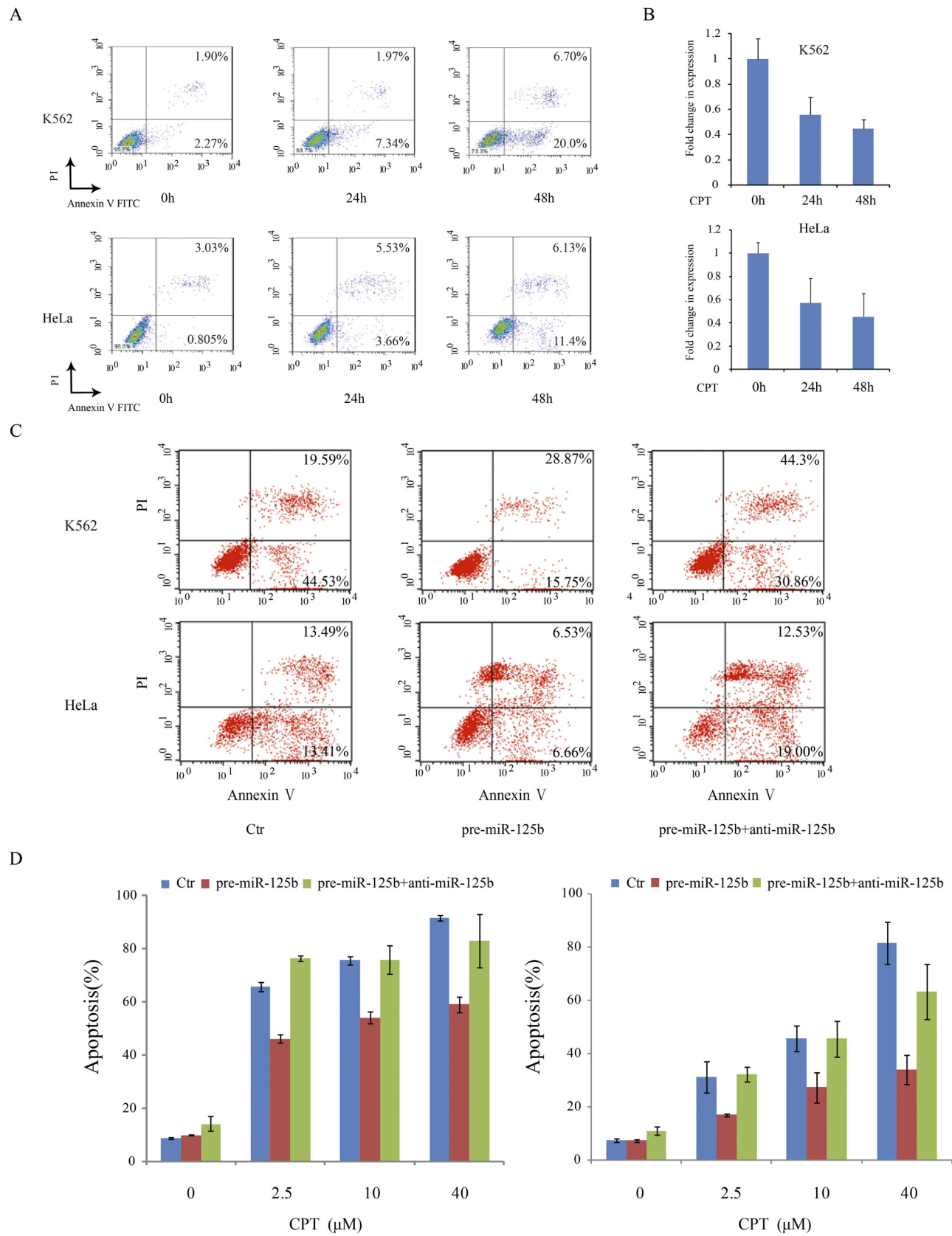
**Luciferase Reporter Assay.** Human embryonic kidney 293T cells at approximately 80% confluence in 48-well plates were cotransfected with 200 ng of the  $\psi$ -CHECK-2-3'UTR (wt) derived reporter vectors and 50 ng of the pcDNA6.2-miR-125b or pcDNA6.2-miR-neg plasmid (miR-neg) expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For K562 and HeLa cells, cells were seeded into 24-well plates (10<sup>5</sup> cells per well). The luciferase activities were measured 48 h after transfection using the dual-luciferase reporter assay system (Promega, Madison, WI). The same protocol was applied for mutated variants of  $\psi$ -CHECK-2-3'UTR (mut) with point mutations at the miR-125b-"seed" sequence. The data were expressed as the relative luciferase activity that was normalized to controls. The 3'UTR sequences of putative mRNA targets containing the putative miR-125b binding site were provided in Supplemental Table 2.

**Statistical Analysis.** Data are expressed as means  $\pm$  S.D. of three independent experiments, each performed in triplicate. Differences between groups were assessed by unpaired, two-tailed Student's *t* test. A statistical difference of *P* < 0.05 was considered significant.

## Results

**MiR-125b Responded to the Induction of CPT in Human Cancer Cells.** CPT is known to exert killing effects by inducing apoptosis in cancer cells (Tan et al., 2009). To reveal whether miR-125b was involved in CPT-induced apoptosis, we investigated the expression patterns of miR-125b during apoptotic stress after CPT treatment. Multiple tumor cell lines were used, including human cervical cancer HeLa cells, human myelogenous leukemia K562 cells, human T cell lymphoblast leukemia CCRF-CEM cells, human non-small-cell lung carcinoma NCI-H1299 cells, human colon carcinoma HCT-15 cells, human cholangiocarcinoma RBE cells, human prostate cancer PC3 cells, and human cervical carcinoma C-33A cells. Cells were treated with 10  $\mu$ M CPT, and apoptotic cells were detected using flow cytometry at 24 and 48 h. The data showed that apoptosis gradually increased in a time-dependent manner (Fig. 1A; Supplemental Fig. 1). At 48 h, the apoptotic rates of CPT-treated cells were varied, compared with K562 and CCRF-CEM cells, the solid cancer cells showed modest changes. In HeLa and K562 cells, the apoptosis rates increased by approximately 16 and 25%, respectively, compared with those of control cells. We detected the expression patterns of miR-125b after CPT treatment using qRT-PCR. We showed that miR-125b was down-regulated in both lines after CPT treatment (Fig. 1B). The expression of miR-125b was similar in p53-null H1299 cells and in normal bronchial epithelial cell HBE (Supplemental Fig. 2A). MiR-125b decreased by approximate 55% in HeLa cells and approximately 55% in K562 cells after CPT treatment compared with that in control cells. Our data revealed that miR-125b expression was responsive to CPT-induced apoptosis in human cancer cells.

To investigate the potential function of miR-125b in CPT-induced apoptosis, we transfected cells with the pre-miR-125b alone or in combination with anti-miR-125b in K562 and HeLa cells. After transfection for 24 h, cells were treated with CPT for 60 h. The apoptotic rates were determined using flow cytometry. The transfection efficiency of miR-125b was measured using the qRT-PCR assay. The results dem-



**Fig. 1.** Involvement of miR-125b in CPT-induced apoptosis in K562 and HeLa cells. **A**, flow cytometric analysis of apoptosis after treatment with 10  $\mu$ M CPT in K562 and HeLa cells at the indicated time points. **B**, miR-125b was down-regulated in K562 and HeLa after treatment with CPT at the indicated time points using SYBR real-time PCR analysis. The relative expression was normalized by U6. **C**, K562 and HeLa cells were cotransfected with pre-miR-125b alone or in combination with anti-miR-125b, and then treated with 0, 2.5, 10, or 40  $\mu$ M CPT for 60 h. A representative experiment (2.5  $\mu$ M CPT) of three performed is shown. The percentage of apoptotic cells is represented in the bar graph from three independent experiments (**D**).



onstrate that the expression of miR-125b increases by more than 25-fold in K562 and 16-fold in HeLa cells after transfection (Supplemental Fig. 2B). We found a significantly lower apoptotic rate in K562 and HeLa cells transfected with miR-125b. After 60 h of CPT treatment, the apoptotic rate in K562 transfected with control was greater than 60%, whereas overexpression of miR-125b suppressed the apoptotic rate to approximately 40% (Fig. 1C). In HeLa cells, overexpression of miR-125b suppressed the apoptotic rate from 31% (miR-NC) to approximately 16%, and the inhibition rate was in a dose-dependent manner with different concentrations of CPT treatment (Fig. 1D). This result was further confirmed in multiple cell lines (Supplemental Fig. 3). The function of miR-125b was also confirmed using colony formation assays. Cells overexpressed miR-125b displayed obvious a higher effect on clonogenicity compared with control (Supplemental Fig. 4). Taken together, miR-125b inhibited CPT-induced apoptosis.

**Bak1, Mcl1, and p53 Are Up-Regulated in CPT-Induced Apoptosis and Are Negatively Correlated with the Expression of miR-125b.** Previous reports have shown that camptothecin induces mitochondrial cytochrome *c* levels and mitochondrial hyperpolarization that precede cytochrome *c* release and the initiation of the apoptotic program (Sánchez-Alcázar et al., 2000). To determine whether CPT induced cell death through the mitochondrial pathway in K562 and HeLa cells and whether miR-125b was involved in this process, the mitochondrial membrane potential was analyzed based on the accumulation of rhodamine 123, which is a membrane-potential indicator, by virtue of its selectivity for mitochondria and its fluorescent properties. The rhodamine 123 fluorescence in mitochondria was decreased in K562 and HeLa cells after CPT treatment, which was analyzed using flow cytometry (Fig. 2A). CPT induced quenching of rhodamine 123 fluorescence, and the rate of fluorescence decay was proportional to the mitochondrial membrane potential. This result shows that CPT triggers the intrinsic pathway of apoptosis. We speculated that the down-regulation of miR-125b may provide opportunities for increasing the expression of its targets to initiate the apoptotic program.

In mitochondrial apoptosis, Bak1, Mcl1, and p53 are the key factors in the mitochondrial pathways of apoptosis (Cory and Adams, 2002; Vaseva and Moll, 2009). In recent years, Bak1 and p53 have been validated as miR-125b targets in several cancers (Shi et al., 2007; Le et al., 2009a; Zhou et al., 2010). In this study, we examined whether CPT regulated Bak1, Mcl1, and p53 expression and was negatively associated with miR-125b expression level. We detected their expression after CPT treatment. After 0, 24, and 48 h of CPT treatment, expression of p53 was detected using Flow cytometry. Mcl1 and Bak1 expression were measured by Western blot analysis. As shown in Fig. 2, B and C, a significant increase in Bak1, Mcl1, and p53 protein levels was detected in response to the treatment. It is noteworthy that the expression levels of three molecules increased in a time-dependent manner and negatively correlated with miR-125b expression, as shown in Fig. 1B. The results suggest that Bak1, Mcl1, and p53 may be the primary targets of miR-125b and indicate the importance of CPT-induced apoptosis via the mitochondrial pathway.

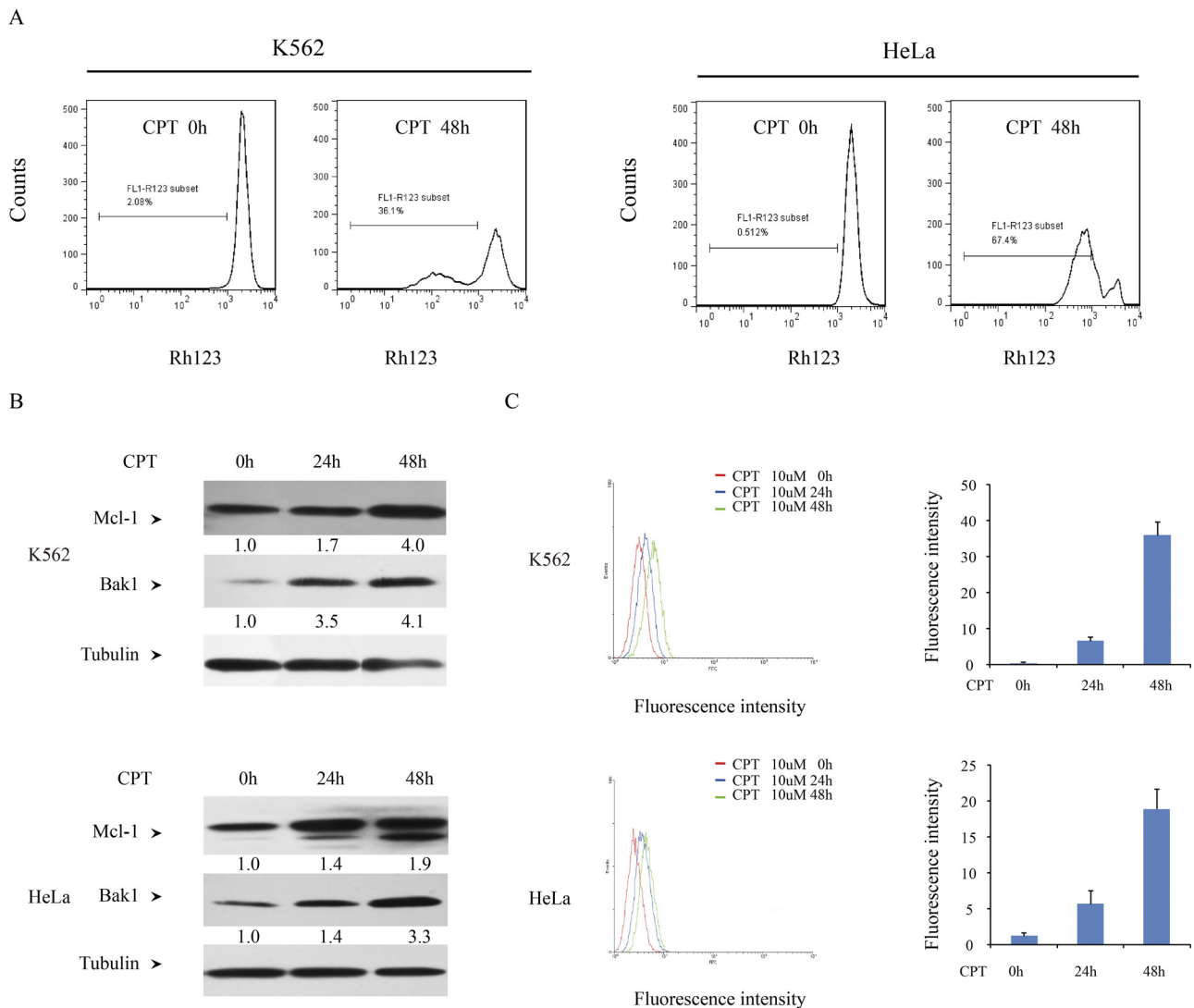
**MiR-125b Mediated p53/Mcl1/Bak1 in CPT-Induced Mitochondrial Apoptosis.** The aforementioned results in-

dicate that miR-125b inhibits CPT-induced apoptosis using a pathway that might be mediated by p53/Mcl1/Bak1. To investigate whether p53, Mcl1, and Bak1 were functional in miR-125b-mediated regulation in CPT-induced apoptosis, a gain-of-function of miRNA method was applied. Both K562 and HeLa cells were transfected with miR-125 mimics and the negative control. After transfection for 24 h, K562 and HeLa cells were treated with CPT for 48 h to induce apoptosis. We measured the effects of miR-125b on p53, Mcl1, and Bak1 protein levels and apoptosis rate. As shown in Fig. 3A, p53, Mcl1, and Bak1 proteins levels were significantly decreased after miR-125b overexpression, and the apoptotic effect was partly prevented compared with miR-NC, as shown above in Fig. 1, C and D. These results suggest that miR-125b represses the expression level of endogenous p53, Mcl1, and Bak1 and, in turn, inhibits CPT-induced apoptosis. To test the functional significance of these observations, we also examined the effect of knockdown of these proteins on CPT-induced apoptosis. RNA interferences against Bak1, Mcl1, and p53 were performed (Supplemental Fig. 5A), K562 and HeLa cells were transfected with scramble siRNA (si-NC) or siRNA (si-Bak1, si-Mcl1, and si-p53), 24 h after transfection, the cells were treated with 2.5  $\mu$ M CPT for 60 h to induce apoptosis. As shown in Fig. 3, B and C, knockdown of Bak1 and p53 inhibited apoptosis, whereas increased apoptosis by Mcl1, consistent with previous reports that Bak1 is proapoptotic whereas Mcl1 is antiapoptotic.

To further confirm whether p53/Mcl1/Bak1 are the direct targets of miR-125b in K562 and HeLa cells, we examined the regulation of endogenous miR-125b at the binding site of the 3'UTRs of p53, Mcl1, and Bak1 mRNA compared with point mutants using dual luciferase reporter gene assays. The results show that miR-125b in K562 and HeLa cells inhibits the luciferase reporter activity in cells that were transfected with the 3'UTRs wild-type seed sequence compared with that in cells transfected with point mutant reporters (Fig. 4A). In addition, overexpression of Bak1 lacking the 3'UTR eliminated the effect of miR-125b in CPT-induced apoptosis (Fig. 4B). Western blotting was carried out with antibody against Bak1 to detect the expression of Bak1 (Supplemental Fig. 5B). We further analyzed the expression of p53/Mcl1/Bak proteins in K562 cells after ectopic expression of miR-125b and treatment with another chemotherapeutic agent doxorubicin. The results showed that miR-125b could also repress the expression level of endogenous p53, Mcl1, and Bak1 under the induction of this therapeutic agent; however, the repression levels were not as significant as that under the induction of CPT (Supplemental Fig. 5C).

Taken together, our results show that p53, Mcl1, and Bak1 are effectors in mitochondrial apoptosis and that miR-125b mediates *p53/Mcl1/Bak1* gene expression in K562 and HeLa cells. Therefore, miR-125b may directly regulate mitochondrial apoptosis via effects on *p53/Mcl1/Bak1* gene expression. In the mitochondrial apoptotic pathway, p53 has been reported to activate Bak1 and disrupt a Bak-Mcl1 complex, which induces apoptosis (Leu et al., 2004). How miR-125b simultaneously targets the *p53/Mcl1/Bak1* complex is not understood yet.

**MicroRNA-125b Directly Targeted the 3'UTR Regions of Multiple Genes in the Mitochondrial Pathway after CPT Treatment.** Because miR-125b suppresses the expression of p53, Mcl1, and Bak1 in CPT-induced mitochon-

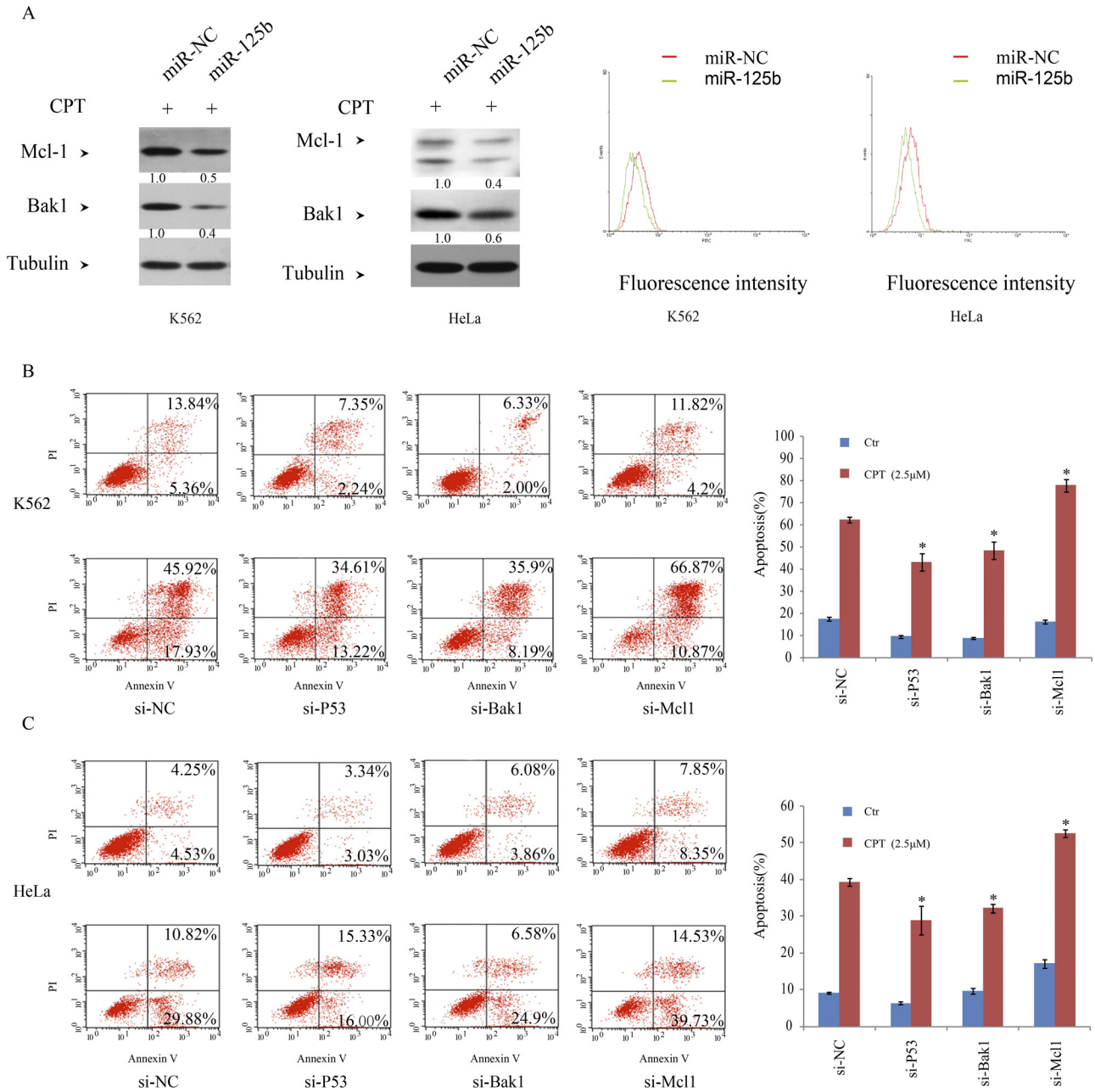


**Fig. 2.** Simultaneous up-regulation of Mcl-1, Bak1, and P53 in K562 and HeLa cells in response to mitochondrial apoptosis that was induced by CPT. Cells were treated with 10  $\mu$ M CPT for the indicated periods of time. **A**, flow cytometric histograms showing mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) in K562 and HeLa cells that were stained with the cationic dye Rh123 at the indicated time points. Histogram plots represent distribution of depolarized mitochondria, the rate of fluorescence decay was proportional to the mitochondrial membrane potential. The experiment was repeated three times with similar outcomes. **B**, Western blot analysis of Mcl-1 and Bak1 expression. **C**, flow cytometry analysis of antibody-stained cells after treatment with 10  $\mu$ M CPT in K562 and HeLa cells at the indicated time points. A shift to the right indicates increased fluorescence intensity. Left, representative results from one of three independent experiments are shown. Right, the flow cytometric analysis was repeated three times and quantified.

drial apoptosis, miR-125b promotes CPT resistance in cancer cells. Most miRNAs target multiple genes that have related functions and thereby exert strong effects on a particular regulatory pathway. To further elucidate the role of miR-125b in CPT-induced apoptosis, the global effects on mitochondrial apoptosis require further examination. Five different target prediction algorithms (PicTar, Target-Boost, TargetScanS, MiRanda, and miRbase) were used to identify potential targets of miR-125b. Hundreds of putative mRNA targets that contained the miRNA response elements (MREs) were compiled with predicted probabilities of preferential conservation using one or more prediction methods. Among these predicted targets, 25 genes were related to mitochondrial apoptosis (Supplemental Table 3) and were chosen for further validation. Because 5 of 25 genes have been reported to be direct targets of miR-125b, the other 20 genes were validated using a luciferase reporter assay. In this assay, individual MREs were cloned into the 3'UTR of a luciferase

reporter gene. The construct plasmids were transfected into human embryonic kidney 293T cells, and the luciferase activity was quantified after 2 days. Figure 5A shows that miR-125b reduced the luciferase activity of the TRIAP1, MYT1, SYVN1, PSMB7, and APC reporters by 30 to 70%. Furthermore, 10 target genes were analyzed for further validation of the specificity with mutated MREs (Fig. 5B). We demonstrated that 10 wild-type UTRs, but not mutated UTRs, were suppressed by miR-125b. Collectively, we validated 20 genes as direct targets of miR-125b in the mitochondrial pathway in addition to previous reports identifying five genes (Shi et al., 2007, 2011; Le et al., 2009a; Xia et al., 2009; Duroux-Richard et al., 2010).

The BCL-2 protein family is functionally inactivated by mutation and/or dysregulation and plays important roles in the initiation and progression of apoptosis. Our results show that several members of the family contain miR-125b-binding sites in their 3'UTRs. Therefore, we investigated the



**Fig. 3.** Knockdown of Bak1, Mcl1, and p53 in CPT-induced apoptosis. A, Mcl-1, Bak1, and P53 were significantly decreased in miR-125b overexpressing K562 and HeLa cells treated with 10  $\mu$ M CTP for 48 h. K562 (B) and HeLa (C) cells were transfected with scramble siRNA (si-NC) or siRNA (si-Bak1, si-Mcl1, and si-p53), 24 h after transfection, followed with 2.5  $\mu$ M CPT treatment for 60 h or not treated. Scramble siRNA served as a negative control. Then the cells were collected for apoptosis analysis by Annexin V staining and flow cytometry. Columns, mean of three independent experiments (right). Cells transfected with si-NC or siRNA (si-Bak1, si-Mcl1, and si-p53) were used as negative controls. \*,  $P < 0.05$  compared with si-NC or siRNA (si-Bak1, si-Mcl1, and si-p53) transfectants.

expression of several BCL-2 protein family genes in K562 and HeLa cells treated with CPT using qRT-PCR. The results show that several BCL-2 protein family genes were up-regulated in CPT-treated cells compared with control cells (Fig. 5C). It is noteworthy that the expression was inversely correlated with miR-125b down-regulation over time (Fig. 1B). These results also support the conclusion that CPT induces apoptosis through a mitochondrial pathway.

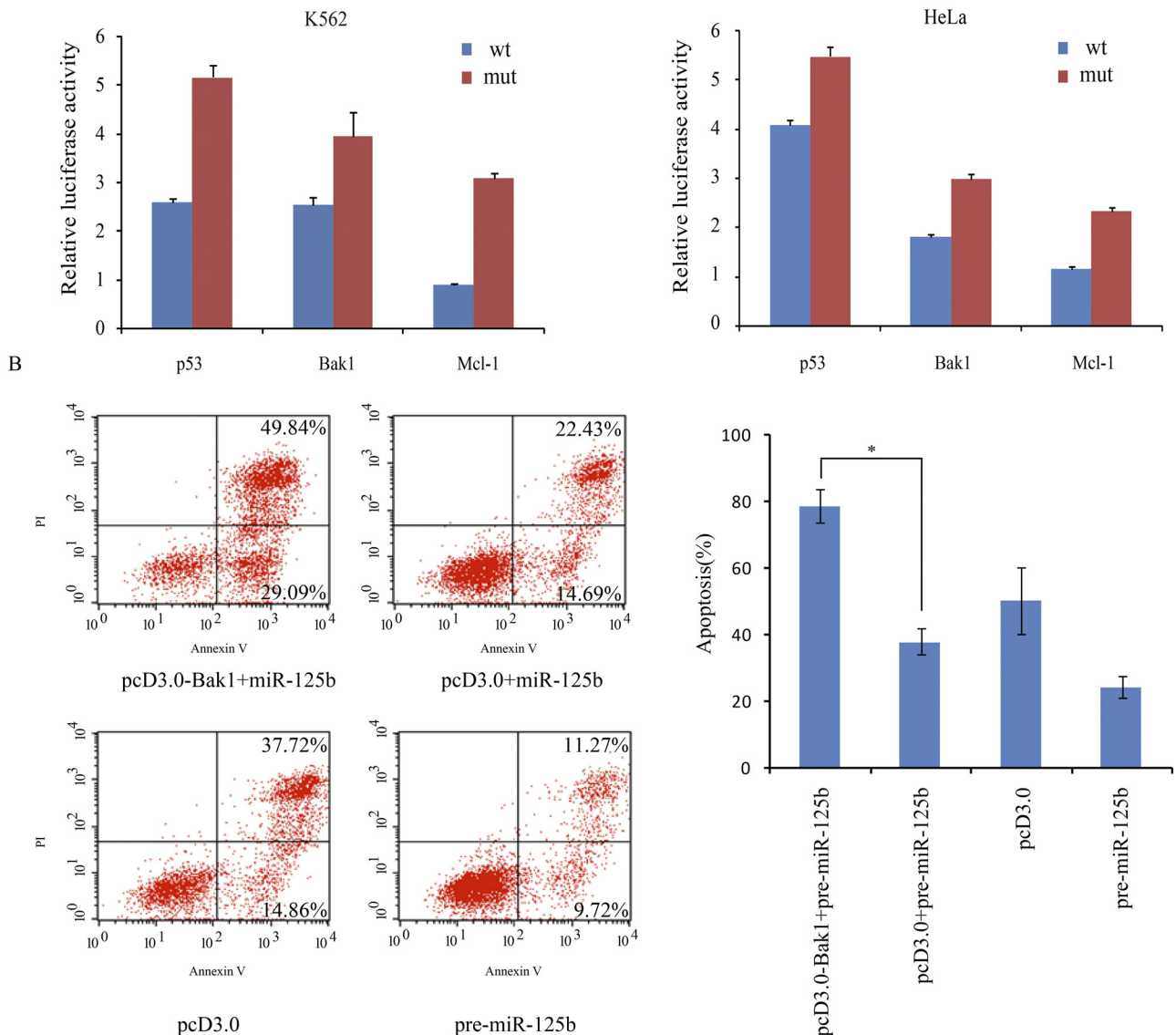
### Discussion

In this study, we identified a role for miR-125b-mediated mitochondrial pathways in CPT-induced apoptosis in cancer

cells for the first time. We demonstrated that miR-125b expression was decreased in CPT-treated K562 and HeLa cells and that miR-125b overexpression significantly repressed CPT-induced apoptosis compared with that in negative controls. We showed that miR-125b was involved in CPT-induced apoptosis via directly targeting the 3'UTR regions of multiple genes in the mitochondrial pathway, including three key proapoptotic factors: Bak1, Mcl1, and p53. These results suggest that the suppression of miR-125b may be a novel approach for the treatment of cancer.

Leu et al. (2004) reported that mitochondrial p53 activates Bak1 and disrupts the Bak-Mcl1 complex to cause apoptosis.

A



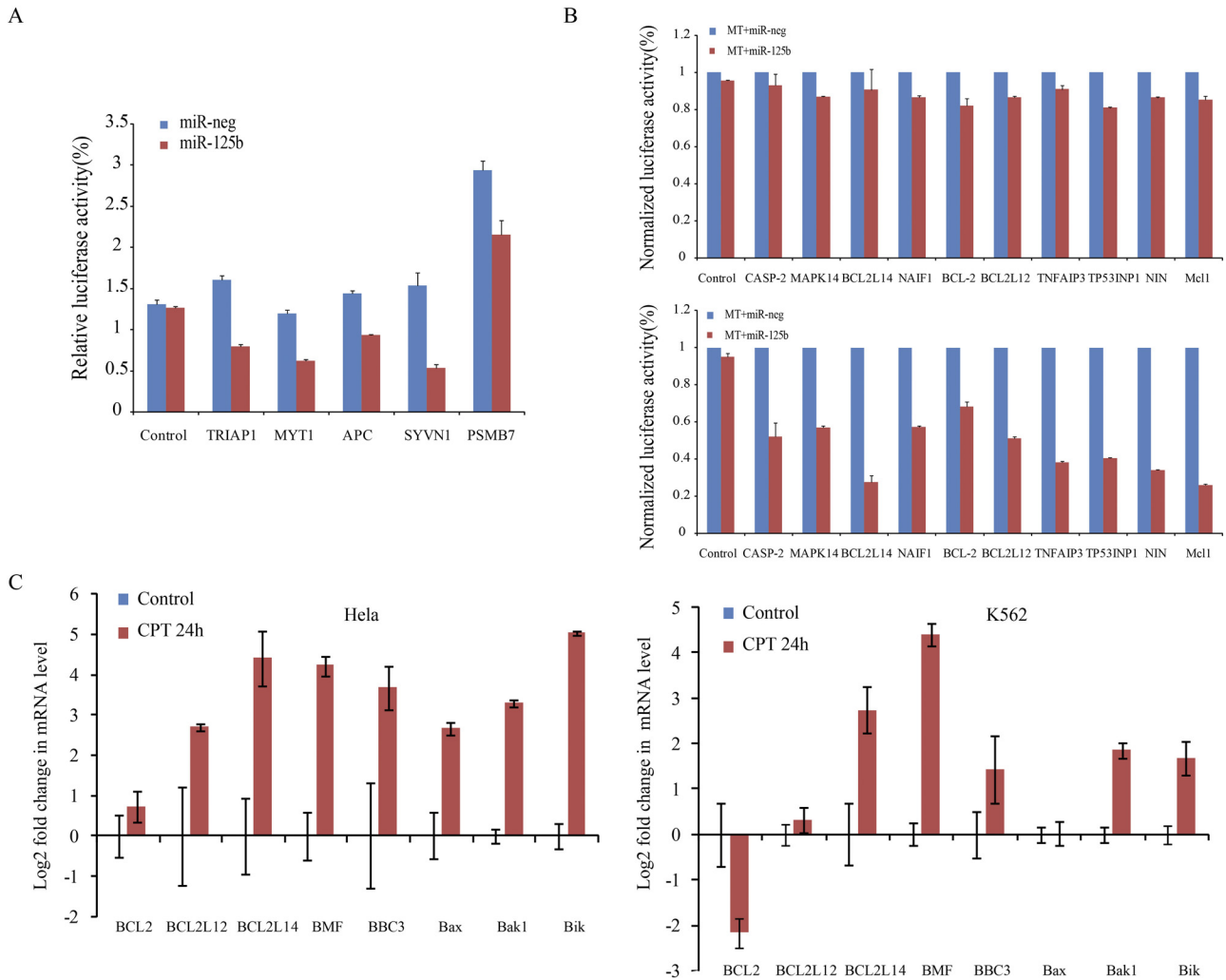
**Fig. 4.** Effects of miR-125b on Mcl-1, Bak1, and P53 in CPT-treated K562 and HeLa cells. **A**, luciferase reporter assay of P53 wt, P53 mut, Bak1 wt, Bak1 mut, Mcl-1 wt, and Mcl-1 mut in K562 and HeLa cells in the presence of miR-125b. The results show that miR-125b markedly inhibits the activity of Mcl-1, Bak1, and p53 reporters. **B**, overexpression of Bak1 lacking the 3'UTR eliminate the effect of miR-125b. K562 cells were cotransfected with 100 nM pre-miR-125b in combination with 2  $\mu$ g of pcDNA3.0-Bak1 or pcDNA3.0. The cells transfected with pre-miR-125b and pcDNA3.0 served as control. Twelve hours after transfection, cells were then followed with 2.5  $\mu$ M CPT treatment for 24 h. Then the cells were collected for apoptosis analysis by Annexin V staining and flow cytometry. The percentage of apoptotic cells is represented in the bar graph from three independent experiments (right).

In this study, we verified that p53, Mcl1, and Bak1 were the direct miR-125b targets and that these genes responded to CPT-induced apoptosis. The proteins p53 and Bak1 are key proapoptotic factors in the mitochondrial apoptosis (Cory and Adams, 2002; Vaseva and Moll, 2009). CPT down-regulates the expression of miR-125b in cancer cells and up-regulates p53 and Bak1 proteins, which induce apoptosis. It is noteworthy that Mcl1, which is an antiapoptotic Bcl2 family member, is required for cell survival (Coultas and Strasser, 2003; Michels et al., 2005; Wong and Puthalakath, 2008). However, Mcl1 is up-regulated in response to DNA-damaging agents, including CPT (Zhan et al., 1997; Mei et al., 2007). CPT-induced Mcl1 up-regulation may represent survival responses to death stimuli. Alternatively, Mcl1 induction may

indicate the molecular basis of the underlying mechanism of acquired resistance to CPT (Mei et al., 2007). We suggest that the induction by CPT represses miR-125b, which leads to up-regulation of p53, Bak1, and Mcl1. Up-regulation of p53 induces oligomerization of Bak1 to promote cytochrome *c* release. Our data revealed that the up-regulation of Mcl1 was moderate, which may indicate the cellular response to CPT-induced stress. Conversely, transfection with miR-125 repressed the expression of p53 and Bak1 and decreased cell apoptosis.

In general, one gene may be repressed by multiple miRNAs, and one miRNA may repress multiple target genes, which results in the formation of complex regulatory networks (Kuhn et al., 2008; Hendrickson et al., 2009; O'Day and Lal,





**Fig. 5.** MiR-125b binds to the 3'UTR of most mitochondrial pathway and its upstream regulator. A, MREs validated by luciferase reporter assay. Repression of luciferase activity is due to the interaction between miR-125b and the predicted MREs in the luciferase-MRE. B, specificity of the miR-125b target site in the 3'UTR validated by mutation and luciferase reporter assay. In mutant reporter constructs, the MRE or a three-mismatch mutation was introduced into the seed region. C, the levels of Bcl-2 family mRNAs were determined using real-time PCR.  $\beta$ -actin was used as an internal control. Results represent the means of three independent experiments.

2010). Le et al. (2009b) have shown that ectopic expression of miR-125b significantly promotes neurite outgrowth and neuronal differentiation via multiple targets. In the present study, we showed that miR-125b interacted with many targets and may mediate mitochondrial CPT-induced apoptosis. This finding is in agreement with a previous report that a single miRNA is likely to target multiple mRNAs (Selbach et al., 2008). More interesting, we found that the targets were pro- and antiapoptotic proteins, indicating versatile functions and a complicated regulatory mechanism of miR-125b in apoptosis. Previous studies have indicated that miR-125b is a tumor suppressor that is down-regulated in breast cancer and targets ERBB2 and ERBB3 (Iorio et al., 2005; Scott et al., 2007). However, Zhou et al. (2010) have not detected miR-125b-induced down-regulation of ErbB2 in breast cancer cells. They demonstrated that miR-125b might act as an oncogene in breast cancer by repressing Bak1 to inhibit apoptosis. We hypothesize that miR-125b may act as a buffer mediator and contributes to cell apoptosis via multiple targets. MiR-125b was down-regulated during apoptosis. The up-regulation of proapoptotic proteins may stimulate cell

apoptosis, whereas up-regulation of antiapoptotic proteins may avoid immediate cell death. This type of model requires further validation.

In conclusion, we identified a role for miR-125b-mediated mitochondrial pathways in CPT-induced apoptosis in cancer cells via miR-125b targeting of the 3'UTR regions of multiple genes, including Bak1, Mcl1, and p53. MiR-125b simultaneously targets a large number of important antiapoptotic genes to ensure a tight control of the apoptotic pathway. It is noteworthy that most, but not all, targets of miR-125b were proapoptotic genes. These findings suggest that miR-125b may repress proteins that comprise the death machinery and promote cellular resistance to apoptotic stimuli.

#### Authorship Contributions

*Participated in research design:* Zeng, X. J. Zhang, and Chen.  
*Conducted experiments:* Zeng, X.-J. Zhang, Lin, Ye, and Feng.  
*Performed data analysis:* H. Zhang and Chen.  
*Wrote or contributed to the writing of the manuscript:* Zeng, X.-J. Zhang, and Chen.



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