

# Suppression of c-FLIP<sub>L</sub> promotes JNK activation in malignant melanoma cells

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**Abstract.** The up-regulation of cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein (c-FLIP) has been reported in various tumor types, and has been previously shown to be associated with the clinicopathological features of melanoma. To assess its potential role in cancer therapy, the present study evaluated the effects of short hairpin (sh)RNAs of different c-FLIP isoforms on cellular proliferation and c-Jun N-terminal kinase (JNK) signaling. Human c-FLIP shRNA plasmids were constructed and transfected into the A875 melanoma cell line. It was observed that c-FLIP shRNA exhibited strong inhibitory effects against the levels of phosphorylated-JNK and inhibited cellular proliferation in A875 cells. Thus, this indicated that c-FLIP long form shRNA serves a specific inhibitory role in cellular proliferation through inducing the activation of the JNK pathway in A875 cells. The present study provided insight into the development of RNAi based therapies for melanoma.

## Introduction

Melanoma is a lethal skin cancer with poor prognosis due to its capacity for early stage metastasis and resistance to chemotherapy, with only 14% of patients with metastatic melanoma surviving for 5 years (1). There is an urgent demand for the development of higher efficacy treatment strategies, and the elucidation of the associated molecular mechanisms.

Nuclear factor- $\kappa$ B (NF- $\kappa$ B) is an essential regulator of gene transcription for numerous genes, including those that are critically involved in apoptosis; and the aberrant regulation of NF- $\kappa$ B an important mechanism involved in the development

of melanoma. Currently, the anti-apoptotic functions of NF- $\kappa$ B are suggested to be predominantly mediated by the upregulation of anti-apoptotic genes including cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-inhibitory protein (c-FLIP), members of the Bcl-2 family and X-chromosome-linked inhibitor of apoptosis (2). In addition to these, recent studies have shown that NF- $\kappa$ B suppresses tumor necrosis factor (TNF)- $\alpha$ -induced cell death by inhibiting prolonged c-Jun N-terminal kinase (JNK) activation (3). However, the detailed molecular mechanisms remain to be fully elucidated.

c-FLIP regulates death receptor-mediated apoptosis (4) and inhibits cluster of differentiation (CD)95- and TNF-related apoptosis-inducing ligand receptor (TRAIL-R)-mediated apoptosis by interfering with the activation of caspase-8 (5). It is expressed as long (c-FLIP<sub>L</sub>), short (c-FLIP<sub>S</sub>) and Raji (c-FLIP<sub>R</sub>) splice variants in human cells (6). Particularly, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> are known to have multifunctional roles in various signaling pathways, including activating and/or upregulating several molecules involved in cytoprotective signaling (7).

In the present study, eukaryotic short hairpin (sh)RNA expression vectors for c-FLIP isoforms were successfully cloned, and their role in cellular proliferation was investigated. In addition, the effect of c-FLIP<sub>L</sub> on JNK signaling was investigated.

## Materials and methods

**Reagents and cell culture.** The following antibodies were used and purchased as indicated: Polyclonal rabbit anti-c-FLIP (1:500; NF6; Enzo Life Sciences, Inc., Farmingdale, NY, USA); polyclonal rabbit anti-phosphorylated (p)-stress activated protein kinase (SAPK)/JNK (Thr183/Tyr185) (1:1,000; 9251; Cell Signaling Technology, Inc., Danvers, MA, USA); polyclonal rabbit anti-SAPK/JNK (1:1,000; 9252; Cell Signaling Technology, Inc.); monoclonal mouse anti- $\beta$ -actin (1:2,000; sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). TNF- $\alpha$  was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The A375, A875 and SK-Mel-1 cell lines were purchased from Shanghai Maisha Biotechnology Co., Ltd. (Shanghai, China). The SK-Mel-28 cell line was kindly provided by Dr Peter M. Blumberg (National Cancer Institute, Bethesda, MD, USA). Cell

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lines were cultured with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humid environment with 5% CO<sub>2</sub>.

**Plasmid constructs for human c-FLIP shRNAs.** To construct shRNAs targeting human c-FLIP (long form accession no. U97074; short form accession no. U97075), the following oligonucleotides were designed according to Tushul's principle (8,9). Pgenesil-1 (10) (Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China) containing complementary DNA of green fluorescent protein and the kanamycin-resistance gene was used for the vector backbone. The control-scrambled shRNA was constructed by the insertion of a similar structure but encoding a nonsense minigene with no homology to any known sequences in human and mouse genomes. Recombinant chimeric plasmids were verified by restriction enzyme analysis and sequencing, and constructs that targets the both isoforms, the long isoform and the short isoform were named as c-FLIP shRNA (target sequence 5'-AACTGCTCTACAGAGTGA GGC-3'), c-FLIP<sub>L</sub> shRNA (target sequence 5'-AAGATGAAG AGCAAGCCCCTA-3') and c-FLIP<sub>S</sub> shRNA (target sequence 5'-ATGCCCATTTGCTCTGATCTGA-3'), respectively. A control scrambled shRNA (target sequence 5'-AATTCTCCG AACGTGTCACGT-3') was also designed.

**Transient transfection of c-FLIP shRNAs.** c-FLIP, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNAs were transfected into A875 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were used for subsequent experiments 48 h following transfection. TNF- $\alpha$  (10 ng/ml) was added to cells (5x10<sup>4</sup> cells/ml) for 4 or 8 h.

**Western blotting.** Cells (5x10<sup>5</sup>) were lysed in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 25 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1 mg/ml leupeptin), the lysate was then centrifuged at 12,000 x g for 15 min at 4°C, the supernatant was collected for western blot analysis as described previously (11). Briefly, protein was separated on 10% SDS-Page gel (Wuhan Boster Biological Technology, Ltd., Wuhan, China) and transferred onto a polyvinylidene difluoride membrane (Pall Corporation, Port Washington NY, USA). Then, 5% skimmed milk was used to block the membrane for 1 h at room temperature. The membrane was then incubated with primary antibodies (listed under reagents) at 4°C overnight, and washed with phosphate buffered saline with Tween-20 (PBST; Beijing Dingguo Biotechnology Co., Ltd., Beijing, China) 5 times for 10 min. Then, the membrane was incubated with the following secondary antibodies at room temperature for 1 h: Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (1:5,000; sc-2313), or donkey anti-mouse HRP-conjugated mouse antibodies (1:5,000; sc-2314) (Santa Cruz Biotechnology, Inc.). Finally, the membranes were washed with PBST 5 times for 10 min. Immunoreactivity was measured using enhanced chemiluminescence (BeyoECL Plus kit; Beyotime Institute of Biotechnology, Shanghai, China), and the band density was

analyzed using ImagePro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

**Determination of cell viability.** Cell viability was determined by 3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, using a kit purchased from Roche Diagnostics GmbH (Mannheim, Germany). Cells (5x10<sup>3</sup>) were plated in 96-well plates and incubated for 48 h and assayed for the number of viable cells according to the manufacturer's instructions. The substrate color development was monitored at 570 nm using a microplate spectrometer (BioTek Synergy HT Multi-Mode Microplate Reader; Bio-Tek Instruments, Inc., Winooski, VT, USA). The assay was repeated three times.

**Statistical analysis.** Data analysis was performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance, followed by Tukey's multiple comparisons test to identify differences between specific groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**c-FLIP expression in melanoma cell lines.** Abundant expression of c-FLIP protein was observed in the A375, A875, SK-Mel-1 and SK-Mel-28 melanoma cell lines, as determined by western blotting. The A875 cell line, which had the highest expression of c-FLIP among the melanoma cell lines tested, was selected for further experiments (Fig. 1).

**Silencing c-FLIP in A875 cells by c-FLIP shRNAs.** The knockdown efficiency of c-FLIP shRNAs was subsequently investigated. The shRNA constructs were transfected individually into A875 cells, and c-FLIP, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNAs all efficiently suppressed the expression of c-FLIP, as shown in Fig. 2.

**c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNA inhibit cell proliferation.** A previous study observed a higher rate of apoptosis in A875 cells transfected with the long or short form of c-FLIP siRNA compared with non-transfected cells or cells transfected with control siRNA. However, the difference between the A875 cells transfected with c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> siRNA was not significant (12). In the present study, alterations in cellular proliferation in A875 cells transfected with c-FLIP<sub>L</sub> shRNA, c-FLIP<sub>S</sub> shRNA, scrambled shRNA and untransfected A875 cells were detected by MTT assay. Cells were cultured for 7 days following transfection, the optical density values were compared. The optical density of A875 cells transfected with the c-FLIP, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNA eukaryotic expression vectors were observed to be significantly reduced compared with the untransfected A875 cells and scrambled shRNA-transfected cells (Fig. 3), suggesting a diminished capacity for cell proliferation. In addition, no significant differences were observed between the c-FLIP, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNA transfected groups.

**TNF- $\alpha$  induces prolonged JNK activation in A875 cells transfected with c-FLIP shRNA.** c-FLIP is a downstream molecule

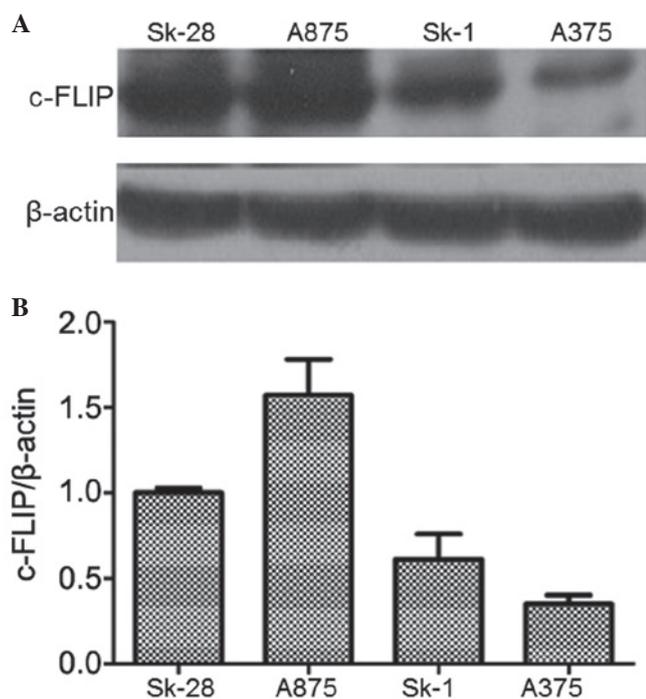


Figure 1. (A) Western blot and (B) quantification of C-FLIP expression in melanoma cell lines. Different melanoma cell lines were subjected to western blotting, which showed that c-FLIP expression was greatest in the A875 cell line. The experiments were repeated 3 times. c-FLIP, cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein; Sk-28, Sk-Mel-28; Sk-1, Sk-Mel-1.

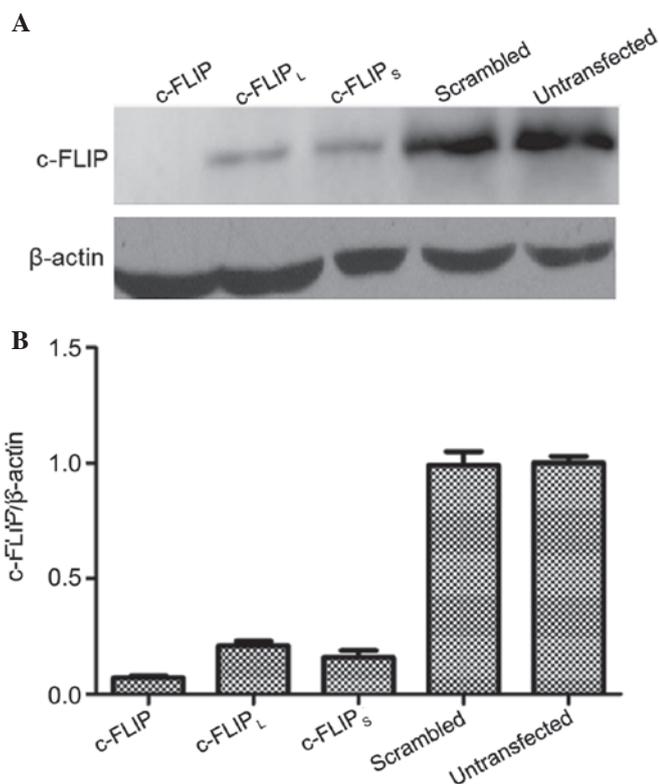


Figure 2. (A) Western blot and (B) quantification of C-FLIP expression measured by western blotting in A875 cells transfected with shRNA. Cells were transfected with different shRNAs for 48 h and subjected to western blot analysis for the expression of c-FLIP. The experiments were repeated 3 times. c-FLIP, cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein; shRNA, short hairpin RNA; c-FLIP<sub>L</sub>, c-FLIP long form; c-FLIP<sub>s</sub>, c-FLIP short form.

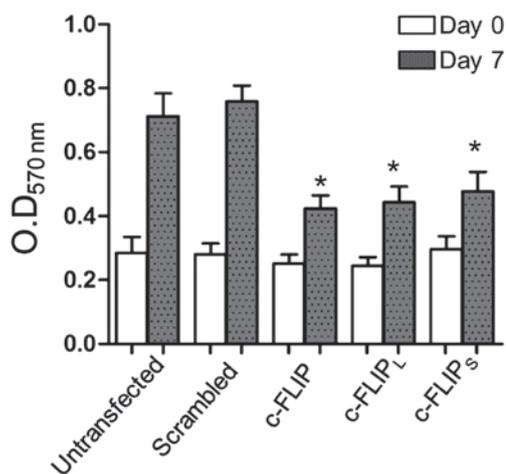


Figure 3. Cell proliferation of transfected and untransfected A875 cells determined by MTT assay. Cells were transfected with different shRNAs for 48 h and incubated for 7 days, and an MTT assay was performed on the day when the cells were plated (day 0) and the 7th day of culture (day 7). OD at 570 nm was acquired and analyzed. \* $P < 0.05$  vs. scrambled group day 7,  $n \geq 5$ . shRNA, short hairpin RNA; OD, optical density; c-FLIP<sub>L</sub>, cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein long form; c-FLIP<sub>s</sub>, c-FLIP short form.

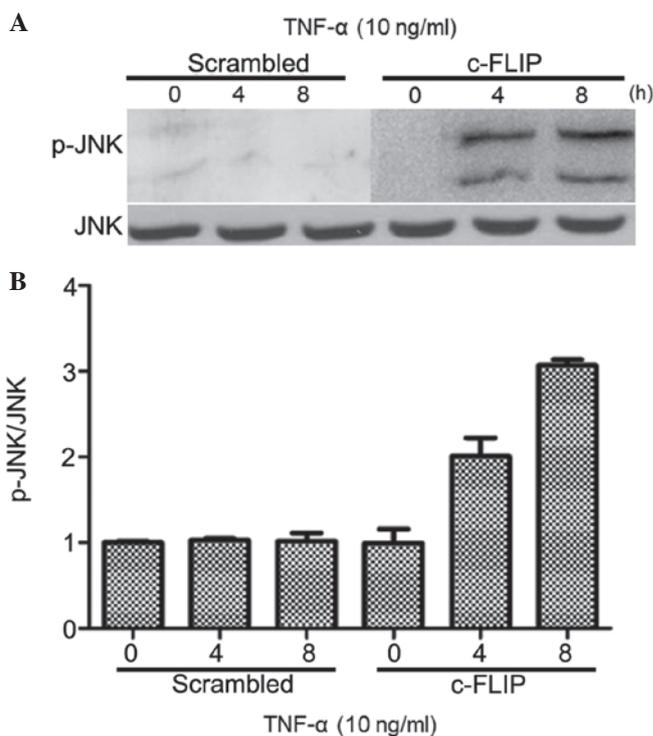


Figure 4. (A) Western blot and (B) quantification of p-JNK expression in short hairpin RNA transfected A875 cells. The transfected cells were treated with TNF- $\alpha$  (10 ng/ml) for the indicated time, and western blotting was used to measure the expression of p-JNK and JNK. The experiments were repeated 3 times. p-JNK, phosphorylated c-Jun N-terminal kinase; TNF, tumor necrosis factor; c-FLIP, cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein.

of TNF- $\alpha$ . and the effect of c-FLIP inhibition on apoptosis, induced by TNF- $\alpha$ , was analyzed. A875 cells growing in the logarithmic phase were transfected with c-FLIP shRNA to target both isoforms, and treated with TNF- $\alpha$  (10 ng/ml) for

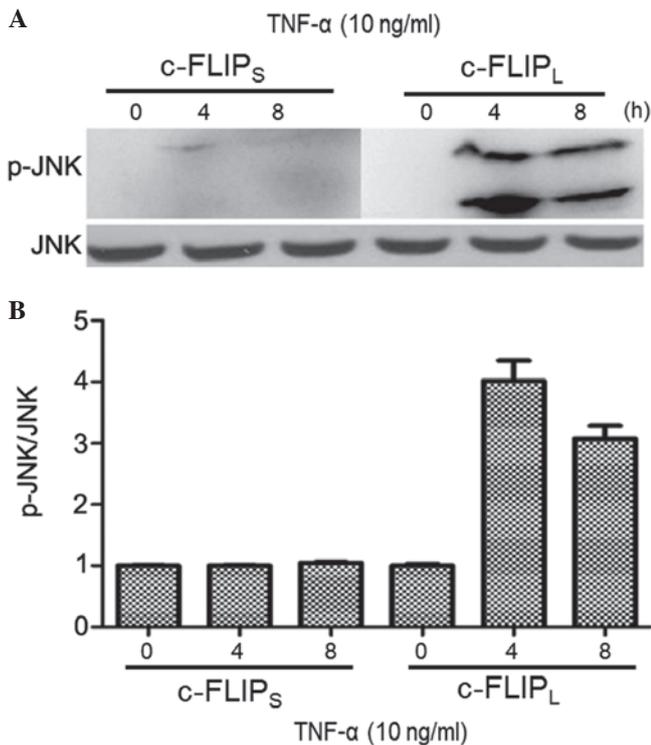


Figure 5. (A) Western blot and (B) quantification of p-JNK expression in c-FLIP<sub>L</sub> or c-FLIP<sub>S</sub> shRNA transfected A875 cells. The transfected cells were treated with TNF- $\alpha$  (10 ng/ml) for the indicated time, and western blotting was used to measure the expression of p-JNK and JNK. The experiments were repeated 3 times. p-JNK, phosphorylated c-Jun N-terminal kinase; TNF, tumor necrosis factor; c-FLIP, cellular Fas-associated death domain-like interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein.

0, 4 and 8 h. Transfection with c-FLIP shRNA induced JNK activation upon treatment with TNF- $\alpha$ , and this activation that was markedly prolonged up to 8 h following treatment (Fig. 4). JNK activation was not observed in cells transfected with scrambled shRNA. These results suggested that increased c-FLIP expression may inhibit JNK activation in A875 cell line.

*TNF- $\alpha$  induces prolonged JNK activation in A875 cells transfected with c-FLIP<sub>L</sub> shRNA but not c-FLIP<sub>S</sub> shRNA.* Next, cells were transfected with c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNAs, and subsequently treated the cells with TNF- $\alpha$  (10 ng/ml). As shown in Fig. 5, TNF- $\alpha$  induced JNK activation in A875 cells transfected with c-FLIP<sub>L</sub> shRNA, however, not in those transfected with c-FLIP<sub>S</sub> shRNA. This suggests that only c-FLIP<sub>L</sub> shRNA is able to induce JNK activation in A875 cell lines, although both c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNA are able to suppress c-FLIP expression.

## Discussion

The expression of c-FLIP has been demonstrated to be a factor involved in the resistance of tumor cells to death ligands such as FasL and TRAIL, and certain reports have demonstrated that downregulation of c-FLIP results in the resensitizing of various types of resistant tumor cells (13-16). These previous studies have delineated the multifunctional role of c-FLIP in

diverse signaling pathways that regulate apoptosis, proliferation, carcinogenesis, and the survival of cancer cells (17,18).

The JNK pathway represents a subgroup of mitogen activated protein kinases (MAPK) that are primarily activated by cytokines and environmental stresses (19). Specific stimuli trigger the activation of MAP3Ks, which then phosphorylate and activate the MAP2K isoforms, MKK4 and MKK7, and subsequently phosphorylate and activate JNK (20). JNKs belong to the superfamily of MAPKs that are involved in the regulation of cell proliferation, differentiation and apoptosis (21). Analysis of the pathways regulated by JNKs have indicated that JNKs are indispensable for both cell proliferation and apoptosis (22). Whether the activation of JNKs leads to cell proliferation or apoptosis is dependent on the stimuli and the cell type involved in such activations (23). There is currently few studies reporting on the regulatory mechanisms of JNK in melanoma. Jørgensen *et al* (24) analyzed sections from 154 primary (93 superficial and 61 nodular melanomas) and 73 metastatic melanomas, together with 34 benign samples using immunohistochemistry. They observed that 35% of the primary and 25% of the metastatic melanoma samples expressed variable levels of p-JNK. By contrast, 73.5% of the benign samples expressed p-JNK (24). Numerous previous studies have indicated that c-FLIP-specific siRNA can induce apoptosis in breast, colorectal, lung and prostate cancer cell lines (25-28). A previous study indicated that c-FLIP<sub>L</sub> enhances tumor cell viability via the activation of extracellular signal-regulated kinase and focal adhesion kinase signaling (29). In addition, previous studies have indicated that overexpression of c-FLIP<sub>L</sub> prevented the proteasomal degradation of  $\beta$ -catenin, thus increasing the  $\beta$ -catenin levels induced by Wnt signaling or cyclin D, thereby promoting tumor cell proliferation and cell cycle progression (30,31). Numerous reports have suggested that the overexpression of c-FLIP<sub>L</sub> promotes tumorigenesis and the invasion of endometrial cancer and cervical cancer (32,33). These studies indicated that c-FLIP has multiple functions in signaling pathways regulating apoptosis, proliferation, tumorigenesis and tumor cell survival. In order to verify whether c-FLIP expression has a regulatory role in the JNK signaling pathway, the expression of c-FLIP was measured in several malignant melanoma cell lines, with A875 cells observed to have the greatest expression and therefore was selected for further experiments. The present study demonstrated that knockdown of c-FLIP or c-FLIP<sub>L</sub> however, not c-FLIP<sub>S</sub> induced prolonged JNK activation, which was consistent with previous studies (34,35). A previous study reported that downregulation of c-FLIP expression was able to induce apoptosis in A875 cells (12). In the present study, using A875 melanoma cells, the effects of c-FLIP, c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNA on the biological characteristics of cells were observed, indicating that different subtypes of c-FLIP shRNA were able to inhibit tumor cell proliferation. These results suggest that high expression of c-FLIP in melanoma cells may not only provide cells with resistance to apoptosis, but also promote cancer cell growth and proliferation. In the current study, both c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> shRNAs demonstrated a marked effect on cell proliferation, however, c-FLIP<sub>S</sub> shRNA did not show any effect on JNK activation. This suggests that c-FLIP<sub>S</sub> may inhibit cellular proliferation through mechanisms other than

JNK activation, highlighting some unsolved mechanistic questions for further research.

To date, several small molecules have been found to lower c-FLIP expression and to sensitize tumor cells to death-receptor mediated apoptosis (4). In addition, the inhibitors of several kinases (MAP/ERK kinase1/2, protein kinase C and phosphoinositide 3-kinase) are reported to lower FLIP expression through blocking the signaling pathways for FLIP transcription. We propose that RNAi-targeted therapies towards key regulatory genes such as c-FLIP will prove useful as a mono-therapy to treat certain types of cancer, in addition to being used in combination with chemotherapy, cytokine therapy or radiotherapy to sensitize drug resistant types of cancer, such as melanoma.

In summary, the present study indicated that c-FLIP<sub>L</sub> serves a role in melanoma proliferation via the JNK pathway. The concept of transfecting a c-FLIP shRNA expression vector to block c-FLIP holds promise as a clinical gene therapy approach for melanoma.

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