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Huiyun Sun for the Master of Science

(name of student) (degree)

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Title: Differential Activation of P53 Downstream Genes in Malignant Melanoma Cells with Different Genetic Contexts

Thesis Chair: _____

Abstract approved: _____

(Thesis Advisor Signature)

This work aims to understand the differential activation of P53 and its downstream genes in response to UV irradiation in different genetic contexts of *braf* and *p53*. Human melanoma SK-MEL-28 (mutant BRAF and mutant P53), A375 (mutant BRAF and wild type P53), WM3211 (wild type BRAF and mutant P53), WM3918 (wild type BRAF and wild type P53), and melanocyte cells were irradiated by UV light. The total RNAs were then extracted from cells that were exposed to the UV irradiation (50 J/m^2) and collected at 6 time points (0, 0.5, 1, 2, 3 and 4 hours) after the UV irradiation. Real-time PCR was employed to measure the expression of the *p53* gene and its downstream genes *mdm2*, *p21*, *p16*, bcl_2 and *bax* at the mRNA level. For all of the four melanoma cell lines, *p53* and *mdm2* expression at the mRNA level were downregulated in response to UV irradiation. After UV exposure, the *p21* gene expression levels were down-regulated, but the relative ratio of *bax*/bcl_2 gene expression level increased, indicating cells prefer to induce apoptosis rather than cell cycle arrest. In the melanocyte,

the transcription level of *p53* slightly declined after UV light irradiation. A decreasing expression level of *mdm2* in both melanocytes and melanoma might predict the same pattern of response to UV light in all melanin-producing cells. The upregulating level of *p16* and decreased ratio of *bax*/*bcl*$_2$ demonstrate that in response to UV exposure, normal melanocyte cells are prone to stop the cell cycle and repair DNA damage instead of triggering apoptosis. Understanding the deregulation of the P53 network in melanoma cells with different genetic contexts will help identify novel drug targets that restore the function of the P53 network.

Keywords: *p53*, BRAF, P53 target genes, melanoma, and UV light

Differential Activation of P53 Downstream Genes in Malignant Melanoma Cells with Different Genetic Contexts

A Thesis

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In Partial Fulfillment

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By

Huiyun Sun

July 2017

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Approved by the Department Chair

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Committee Member

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Committee Member

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Dean of the Graduate School and Distance Education

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PREFACE

The organization of this thesis references the publication style of *Cell Death & Disease*.

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Introduction

The P53 protein is a key transcription factor which serves as a loyal watchman to monitor the integrity and function of human cells [1]. *p53* is a tumor suppressor gene whose mutations are associated with numerous human cancers [2, 3, 4, 5]. In normal cells, the P53 protein is found at a low level owing to the ubiquitin-dependent degradation mediated by the feedback loop of MDM2 [6, 7, 8]. When the cells are confronted with physiological stress such as UV irradiation or hypoxia, the P53 protein is stabilized and accumulated via phosphorylation modification, which enables the formation of the homotetrameric complex of P53 and allows the P53 protein to bind to DNA and regulate expression of its downstream genes [9]. On the P53 protein, residues between 98 and 292 are the core DNA-binding domain. The DNA sequence that P53 can bind to is a consensus sequence, consisting of two copies of 10 base pairs of motif 5'-PuPuPuC(A/T) (T/A)GPyPyPy - 3' separated by 0-13 bp [10, 11]. Approximately 80% of the p53 mutations are missense mutations and most of them localize at this DNA-binding domain [12]. The mutant P53 can also form heterotetramers with wild-type P53, which acts in a dominant-negative manner to greatly impair the transactivation of P53 [13, 14].

By matching the common DNA binding site of P53, about 50 potential P53 target genes were identified and classified into two types based on their biological effects [15, 16]. One is related to cell-cycle arrest, such as *mdm2* [6], *p21* [17] and *14-3-3σ* [18], and *GADD45* [19]; the other is related to apoptosis, such as *bax* [20], *PUMA* [21] and *NOXA* [22]. Differences between thees two categories of P53 binding DNA sequences are the sequences

interspersed between the two half-sites of the P53 DNA binding site and the number of the mismatches with the P53 consensus DNA binding site [15]. The post-transcriptional modification patterns of P53, which stabilize P53, are varied. Modifications of the N-terminus of P53 are associated with its transactivation ability and the binding with the negative regulator MDM2 and the phosphorylation of the C-terminus relates to the ability of P53 to recognize DNA damage and bind to DNA [23]. Different types and intensity of physiological stimuli induce unique phosphorylation patterns of P53 by distinct kinases and phosphatases determining different cellular responses [24]. The P53 will stop cell cycle and repair DNA damage when those genetic damages are still repairable. For example, after ionizing radiation, phosphorylation of Ser20 on the N-terminus of P53 by Chk2 will activate P21 to induce cell cycle arrest [25]. When cells experience massive irreparable DNA damage, P53 will regulate the gene expression involved in apoptosis. For instance, phosphorylation of Ser46 on P53 after severe DNA damage upregulates the TP53AIP1 gene, encoding the p53-regulated apoptosis-inducing protein 1 (p53AIP1) which induces apoptosis [26].

Melanoma is the deadliest type of skin cancer, originating from pigment-producing melanocytes. In 2016, 68,480 cases of melanoma were estimated to be diagnosed [27]. Even though melanoma accounts for only a small percentage of skin cancers, it causes the most deaths relative to all skin cancers [28]. UV irradiation is the primary factor in melanoma initiation, progression, and metastasis [29, 30]. The BRAF (V600E) mutation, one of the driving mutations, is found in approximately 50% of melanomas [31]. These mutations enable the destabilization of the inactive form of BRAF

protein which constitutively activates the Ras-Raf-Mek-Erk cell growth intracellular signaling pathway, resulting in uncontrolled cell growth of melanoma [32]. Other mutations, including PTEN, c-kit, CDK4, cyclin D1, p53, MDM2, PI3Ka and AKT3, are involved in melanogenesis as well [33].

Surgery, radiation therapy, chemotherapy, immunotherapy and targeted therapy are currently the main treatments for melanoma [34, 35]. Surgery is mostly effective for the non-metastatic melanoma. Radiation therapy works on all types and stages of melanoma, albeit lacking specificity. Dacarbazine, an FDA-approved chemotherapy, has been used in cases of advanced-stage melanoma [36]. Dacarbazine is an alkylating agent that adds alkyl groups to DNA, interrupting DNA replication in cancer cells [36]. Due to the cytotoxicity of dacarbazine to normal cells, chemotherapy is gradually replaced by targeted therapy, although it is still an option when the patients do not harbor specific mutations.

In targeted therapy, a BRAF targeted inhibitor called vemurafenib (PLX4032) functions to stabilize the inactive conformation of mutated BRAF tyrosine kinase but not the non-mutated normal BRAF. Vemurafenib was approved by the Food and Drug Administration (FDA) in 2011 to treat metastasized melanoma [37]. This targeted drug increases the median overall survival and progression-free survival for the melanoma patients with mutated hyperactive BRAF [38]. However, acquired resistance to this targeted therapy is almost inevitable in melanoma patients receiving vemurafenib treatment owing to the reactivation of the MAPK signaling pathway or induction of other growth signaling

pathways, such as upregulation of the COT-ERK pathway [39], the RTK-N-Ras pathway [40], and the IGF-1R/PI3K pathway [41]. To overcome the drug's resistance to BRAF inhibitors, the combination therapies of BRAF inhibitors and other kinase inhibitors have been exploited [42, 43].

Immunotherapy has been emerging as a promising strategy in fighting advanced melanoma. In immunotherapy, IL-2 (interleukin-2), a T cell growth factor, had been employed to treat metastatic melanoma with a 5 to 10 % rate of complete cancer regression [44]. Nevertheless, less than 10% of patients with metastatic melanoma are eligible to receive IL-2 because of the cytotoxicity of the high IL-2 dosage and the immune-related adverse events (irAEs) associated with this drug [44]. In 2011, another immunotherapy drug, ipilimumab, was approved by the FDA for clinical trials [45]. Ipilimumab is a monoclonal antibody reactive with the inhibitory CTLA-4 cell-surface molecules, activating T lymphocytes and enhancing its antitumor immune response [45]. Melanoma patients treated with ipilimumab experienced improved overall survival [46]. The combination of ipilimumab with other therapies (such as dacarbazine) is continually being evaluated to potentially increase clinical benefits in treating advanced melanoma [47].

Since melanoma is the cancer of which the incidence has continued to increase, finding new genetic targets for treating melanoma is now imperative. As a mutation hotspot in many types of cancer, *p53* is mutated in some melanomas [33]. Even though the research based on the DNA sequencing of *p53* surprisingly reveals that the mutated *p53* occurs at a lower frequency in melanoma compared with other types of skin cancers [48, 49],

a higher rate of *p53* gene aberration has been found in metastatic melanoma [48]. In addition, a high level of P53 expression is found in melanoma regardless of the mutation status of the *p53* gene [48, 50]. It was also found that P53-target-genes involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma, and this abnormal regulation network of P53 may contribute to the progression of melanoma [50, 51].

UV light can trigger cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs) on DNA structures [52]. Such DNA damage can activate ATM, ATR, CDK, JNK and other kinases to phosphorylate and stabilize P53, inducing the transactivation of the P53 downstream genes involved in nucleotide excision repair (NER) or apoptosis [24]. All of these responses are usually detected within 8 hours after UV treatment. To elucidate the effects of different genetic contexts on the anomalous P53 regulation network, we performed experiments to identify different expression patterns of genes under the control of the P53 in several melanoma cell lines with different gene mutations in response to UV irradiation, aiming to examine how the P53 network is dysregulated in melanoma cells and to identify new gene targets for treating melanoma cells. Four melanoma cell lines were used in this research, two with a mutant form of P53 and two with a mutant form of BRAF. Significantly, mRNA levels of *p53* and *mdm2* were down-regulated in all the melanoma cell lines with different genetic contexts after UV treatment. The decrease of *p21* expression and increase of the ratio of *bax*/*bcl*$_2$ indicate that the melanoma prefers to induce apoptosis instead of cell cycle arrest in response to UV irradiation. After UV exposure in melanocyte cells, the increasing mRNA level of *p16* and decreasing ratio of *bax*/*bcl*$_2$ illustrate normal melanocyte cells are

inclined to arrest cell cycle rather than triggering apoptosis. Understanding the different P53 network regulation in response to UV-damage between melanoma cells and melanocyte cells is a key to find the crucial mechanism involved in maintaining the integrity of the cells.

Materials & Methods

Cell lines maintenance

The human melanoma cell lines SK-MEL-28, A375, WM3211, WM3918, and melanocytes were used in this research. The gene context of different cell lines are listed in Table 1. The melanoma cell A375 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with fetal bovine serum (10%) (FBS; Hyclone, Logan, UT) and penicillin/streptomycin (1%) (Fisher Bioreagents, Pittsburgh, PA). The melanoma cell line SK-MEL-28 was cultured in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) supplemented with FBS (10%), and penicillin/streptomycin (1%). The melanoma cell line WM3211 was cultured in MCDB 153 medium (Sigma-Aldrich, St. Louis, MO) supplemented with FBS (2%) and 0.1% penicillin/streptomycin. The melanoma cell line WM3918 was cultured in MCDB 153 medium (Sigma-Aldrich, St. Louis, MO) supplemented with FBS (10%) and 0.1% penicillin/streptomycin. Melanocytes were cultured in Medium254 (Thermo scientific, Pittsburgh, PA) supplemented with PMA-Free Human Melanocyte Growth Supplement-2 (HMGS-2) (Thermoscientific, Pittsburgh, PA). 0.25% Trypin-EDTA solution (Sigma-Aldrich, St. Louis, MO) was diluted into 0.05% using Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO). Soybean Trypsin Inhibitor (Thermoscientific, Pittsburgh, PA) was dissolved in HBSS to a concentration of 0.5mg/mL. 0.05% trypsin and 0.5mg/ml trypsin inhibitor were used to detached melanocyte cells from the culture flasks.

Cells were maintained in exponential growth in the presence of 5% CO^2 at 37°C humidified atmosphere in the atmosphere incubator (Thermoscientific, Pittsburgh, PA). The medium was replaced depending on the growth of different type of cell lines, and cells were passed using 0.05% Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO).

	braf	*p53*	*ras*	*pten*
SK-MEL-28	V600E	Mutated	wt	wt
A375	V600E	wt	wt	wt
WM3211	wt	Mutated	Not determined (ND)	wt
WM3918	wt	wt	ND	wt

Table 1. The gene context of each melanoma cell line used in this project.

UV irradiation

For each cell line, the cells were first passed to 6 Nunc™ Cell Culture/Petri Dishes (Thermoscientific, Pittsburgh, PA) with 80% confluency for each plate. The cells were exposed to UV light with a fixed UV dosage that was calculated at an intensity of 50 J/m^2, which is an appropriate dose that is able to activate the P53 protein [53]. After 0.5h, 1h, 2h, 3h and 4h, the total RNA was extracted to measure the expressions of different genes at the mRNA level. Also, another group of cells were irradiated with the

same intensity, and the protein was extracted to measure the p53 expression at the protein level after 0h, 2h, 4h, and 8h.

RNA extraction

Total RNA was extracted from melanoma cell lines and melanocytes using GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturers' protocols. Purified RNA was stored at –80 °C.

The purity and integrity of the isolated RNA was assessed with a NanoDrop 2000C spectrophotometer (Thermoscientific Inc., Wilmington, Delaware) and by electrophoresis on a 1% denaturing agarose gel.

1% Denaturing agarose gel electrophoresis

5 mL 10X MOPS solution, 40 mL distilled water and 0.45 g agarose were added to a flask. A bottle cap was used to cover the mouth of the flask. This was microwaved for 30 s to 40 s to melt the agarose. 5 mL 37% formaldehyde and 5 µl 1% Ethidium bromide were added to the flask. The gel was gently poured using a comb that would form wells large enough to accommodate at least 25 µL. A gel formed in about 1 h. The gel was placed in a tank and enough 1X MOPS running buffer was added to cover the gel by a few millimeters. After comb was removed, 1 µg of RNA sample with 1 X 1 µL

loading stain was added to each well. The gel was loaded and electrophoresed at a constant voltage of 120 V for 25 min.

Real-time qPCR

Two step reactions were prepared as directed by the manufacturers' instructions (GoTaq® 2-Step RT-qPCR System). Total RNA (300 ng) was reverse transcribed to generate cDNA using the GoScript™ Reverse Transcription System (Promega, WI) with the following program: 25°C for 5 minutes, 42°C for 1 hour, 70°C for 15 minutes and hold at 4°C. A Mastercycler Realplex 2 real-time PCR system was used for thermocycling with the following conditions: 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, melting curve set by the software spontaneously and hold at 4°C. All reactions were performed in triplicate, with no-template-controls and no-reverse-transcriptase controls run for each RNA sample. The component for each reaction was as follows: 12.5 μL GoTaq® qPCR Master Mix, 2X, 0.05 μL Forward qPCR primer, 0.05 μL Reverse qPCR primer, 7.4 μL Nuclease-Free Water, and 5 μL cDNA template. For the no-template controls and no reverse-transcriptase controls, the nuclease free water and total RNA were added respectively instead of the cDNA template. The expression of the following transcripts was analyzed: *p53* (F: 5'-GCTCGACGCTAGGATCTGAC-3'; R: 5'-GCTTTCCACGACGGTGAC-3'), *mdm2* (F: 5'-CCTGATCCAACCAATCACCT-3'; R: 5'-TGTTGTGAAAGAAGCAGTAGCA-3'), *p21* (F: 5'-TGTCCGTCAGAACCCATGC-3'; R: 5'AAAGTCGAAGTTCCATCGCTC-3'), *bax* (F: 5'-AACCATCATGGGCTGGA-3'; R: 5'-CGCCACAAAGATGGTCAA-3'), *bcl*$_2$ (F: 5'-

GGTGGGGTCATGTGTGTGG-3'; R: 5'-CGGTGCAGGTACTCAGTCAATCC-3'), *p16* (F: 5'-GATCCAGGTGGGTAGAAGGTC-3'; R: 5'-CCCCTGCAAACTTCGTCCT-3'), *gapdh* (F: 5'-GAAGGTGAAGGTCGGAGTCAAC-3'; R: 5'-CAGAGTTAAAAGCAGCCCTGGT-3'), *hrpt-1* (F: 5'-TGACACTGGCAAAACAATGCA-3'; R: 5'-GGTCCTTTTCACCAGCAAGCT-3'), and *β-actin* (F: 5'-TCACCCACACTGTGCCCATCTACGA-3'; R: 5'-CAGCGGAACCGCTCATTGCCAATGG-3') (Integrated DNA Technologies, Coralville, Iowa). The relative expression of the gene of interest was normalized to the internal control genes and expressed as the fold change calculated using the 2-ΔΔCt method.

Western Blotting

Cells were trypsinized, washed with PBS for 1 time, and then lysed in ice-cold lysis buffer (0.1 ml per 1x106 cells) (Cell Signaling Technology, Danvers, MA) mixed with protease inhibitor (1:100) (Cell Signaling Technology, Danvers, MA) for 30 min at 4°C. The protein in the supernatant was obtained after centrifugation at 13,000 rpm for 20 min at 4°C. The concentration of the protein was detected by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). The protein samples were boiled with Laemmli 2X sample buffer (Bio-Rad, Hercules, CA) (mixed with 5% 2-Mercapto-ethanol (β-ME) (Sigma-Aldrich, St. Louis, MO) at 1:1 ratio for 10 minutes.

Protein samples were loaded into the well of the Any kD Mini-PROTEIN TGX Stain-Free Gels (Bio-Rad, Hercules, CA) according to the concentration, and 6 μL Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA) was loaded as well. The gel was electrophoresed in the 1X running buffer for 2 hours to which an electrical field (Fisher Scientific FB3000: constant voltage 90V) was applied. The protein was transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) under 300 mA constant current for 1.5h in 1X transfer buffer. The membrane was blocked with 5% non-fat milk in TBST for 1 h at room temperature with constant agitation. This membrane was then immunoblotted with primary antibody for rabbit anti-GAPDH and anti-P53 (Cell Signaling Technology, Danvers, MA) with a dilution of 1: 10000 and 1: 1000 respectively, at 4 °C overnight. The membrane was incubated with HRP-linked secondary antibody (1: 2500) for 2 h at room temperature. The membrane was then incubated with Western HRP Substrate (0.5 ml reagent A and 0.5 ml reagent B) for 5 min in the dark at room temperature. The image was developed by the FluorChemTM E system (Protein Simple, Santa Clara, CA). The intensity of the band was analyzed by the ImageJ [54]

Statistical analysis

All values were represented as mean ± standard deviation (SD) from three replicates. Three independent experiments were carried out in the research.

Results

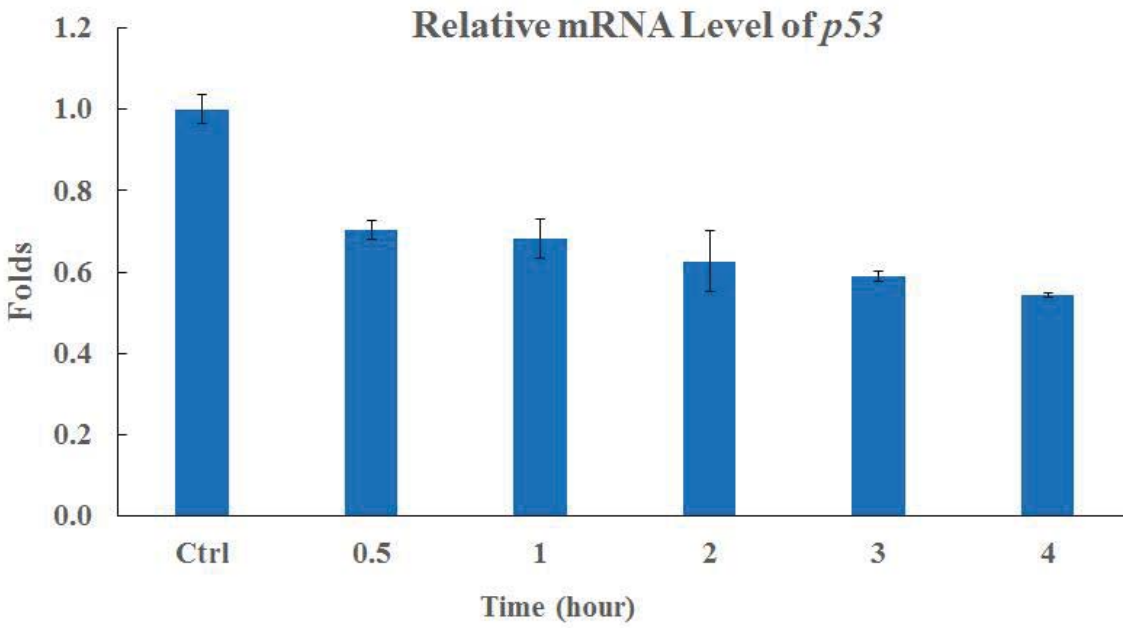
1. mRNA levels of genes involved in cell cycle arrest genes except for *p16* decreased, while mRNA levels of apoptotic genes increased after UV irradiation in mutant BRAF and wild-type P53 human melanoma cell line (A375 cell line).

A375 is a metastatic malignant melanoma cell line with $BRAF^{V600E}$ mutant and wild-type P53. The mRNA levels of the P53 downstream genes were measured using real-time quantitative PCR. Compared with the control group, relative mRNA level of *p53* dropped approximately 30% and kept decreasing starting from the time point of 0.5-hour after UV radiation (Figure 1a). For genes involved in cell cycle arrest, relative expression at mRNA level of *mdm2* decreased in response to UV light (Figure 1b), and expression of *p21* lowered after UV irradiation but started to increase at the time point of 1 hour after UV treatment (Figure 1c). For the *p16* gene, the mRNA level was increased from 0.5- to 3-hours and then began to decline at the time point of 4-hours (Figure 1d). For the genes involved in apoptosis, the expression levels of bcl_2 and *bax* genes both increased (data not shown), whereas the relative ratio of *bax*/bcl_2, which determines the induction of apoptosis, rose after UV light treatment, except for a transient reduction at the time point of 2-hours (Figure 1e).

Figure 1. Relative mRNA levels of *p53* and P53 downstream genes in A375.

After UV irradiation, relative mRNA levels of *p53* (1a), *mdm2* (1b), *p21* (1c), *p16* (1d), and the ratio of *bax*/*bcl*$_2$ (1e) in A375 were measured by real-time qPCR. Each experiment was repeated two times with quadruplicate reactions for each gene. The results are represented as mean±SD.

a.


Relative mRNA Level of p53
Folds
1.2
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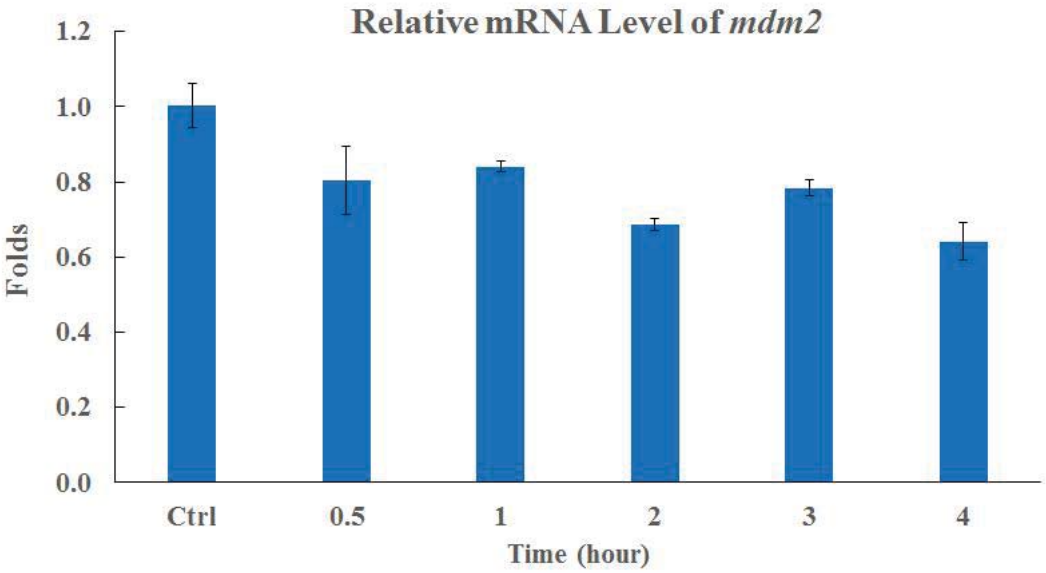
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

b.


Relative mRNA Level of *mdm2*
Folds
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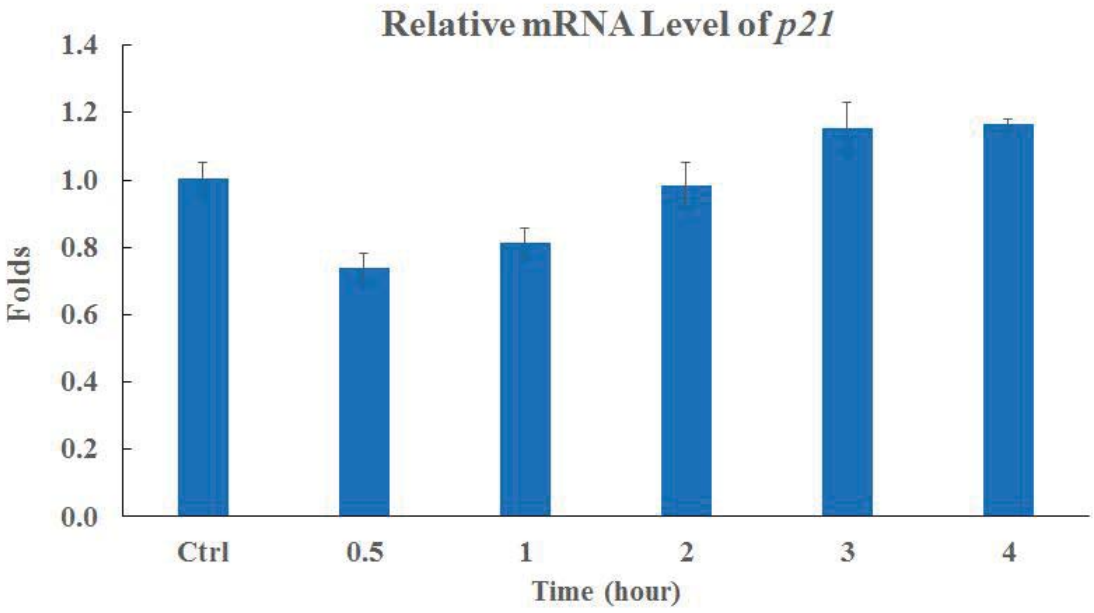
Ctrl
0.5
1
2
3
4
Time (hour)

c.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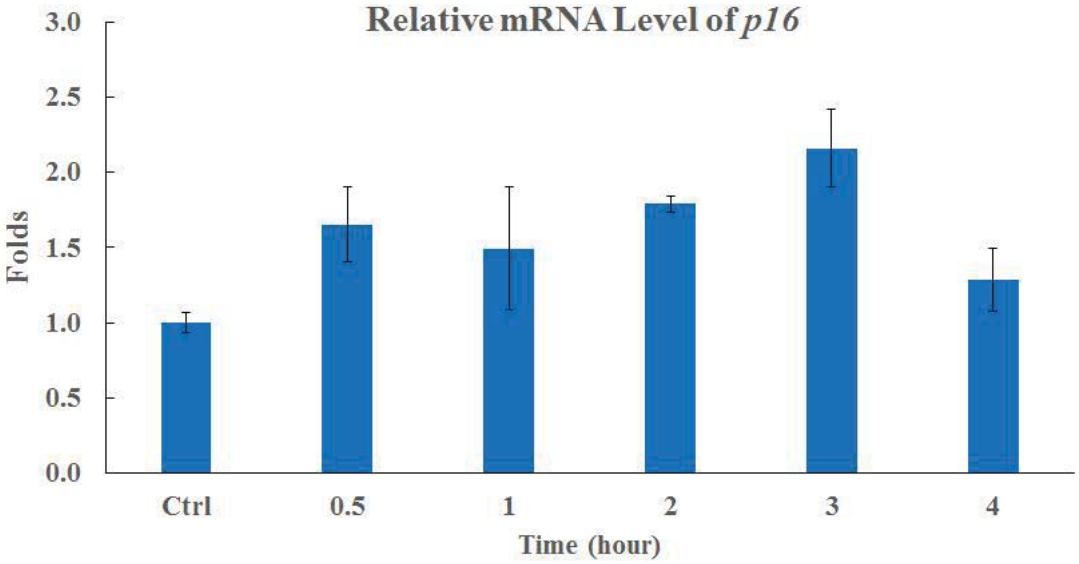
0.5
1
2
3
4
Time (hour)

d.


Relative mRNA Level of *p16*
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Folds
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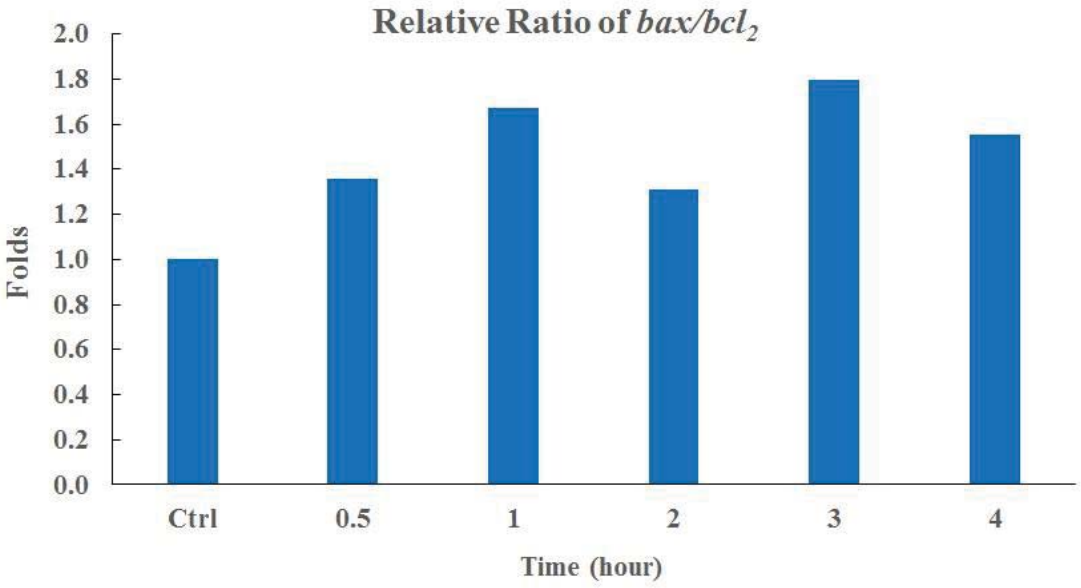
0.5
1
2
3
4
Time (hour)

e.


Relative Ratio of bax/bcl2
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1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

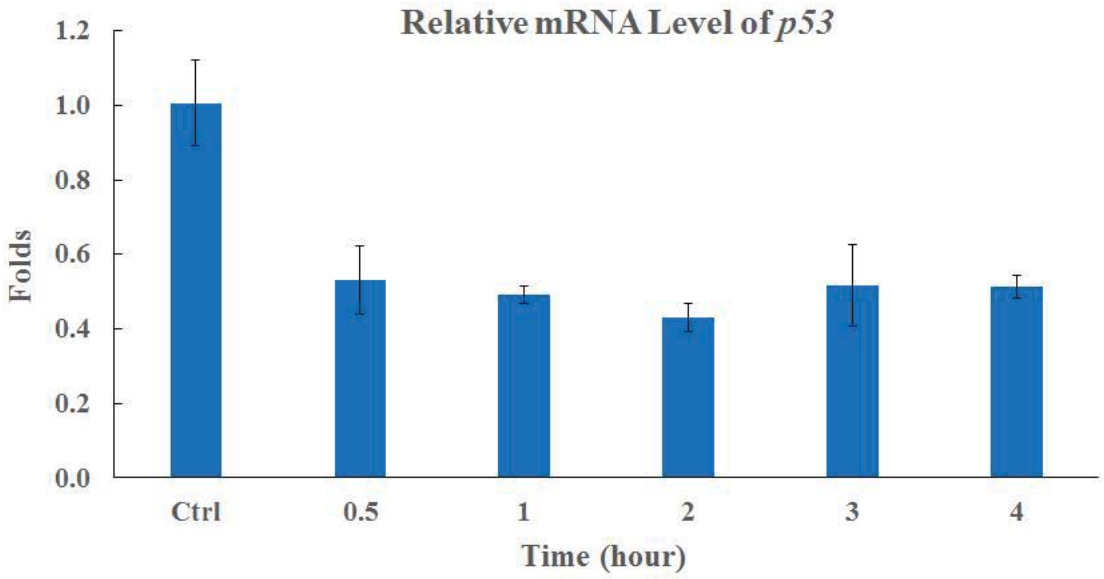
2. mRNA levels of genes involved in cycle arrest genes declined, while mRNA levels of apoptotic genes increased after UV irradiation in BRAF and P53 mutant human melanoma cell lines (SK-MEL-28 cell line).

The genetic context of the metastatic malignant melanoma cell line SK-MEL-28 related to BRAF and P53 is $BRAF^{V600E}$ and $P53^{L145R}$. The expression levels of P53 network downstream genes were examined in SK-MEL-28. After UV light exposure, the relative mRNA level of *p53* declined about 50% and remained in a relative stable status (Figure 2a). Relative expression at mRNA level of *mdm2* decreased after the UV irradiation and the transcription level began to recover 1 hour after UV irradiation (Figure 2b). The expression level of *p21*, encoding a cell cycle inhibitor, slowly decreased in response to UV light, reached the lowest level at the time point of 2-hour, and then increased to a level which was higher compared to the control group (Figure 2c). The mRNA level of *p16*, coding for another cell cycle inhibitor, abruptly lowered after the UV irradiation, and began to rise at the time point of 2-hours (Figure 2d). Bcl_2, an apoptosis inhibitor, was expressed at a reduced level; and *bax*, an apoptosis promotor, was expressed at a higher level, resulting an increase of the relative ratio of bax/bcl_2 (Figure 2e).

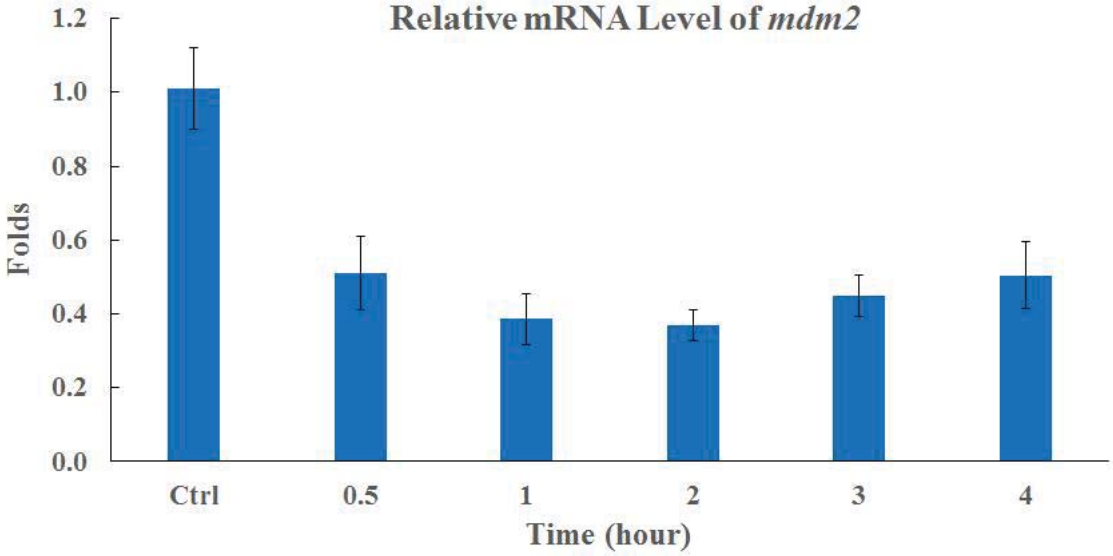
Figure 2. Relative mRNA levels of *p53* and P53 downstream genes in SK-MEL-28.

After UV irradiation, relative mRNA levels of *p53* (2a), *mdm2* (2b), *p21* (2c), *p16* (2d), and the ratio of *bax*/*bcl*$_2$ (2e) in SK-MEL-28 were measured by real-time qPCR. Each experiment was repeated two times with quadruplicate reactions for each gene. The results are represented as mean±SD.

a.


Relative mRNA Level of p53
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Folds
Ctrl
0.5
1
2
3
4
Time (hour)

b.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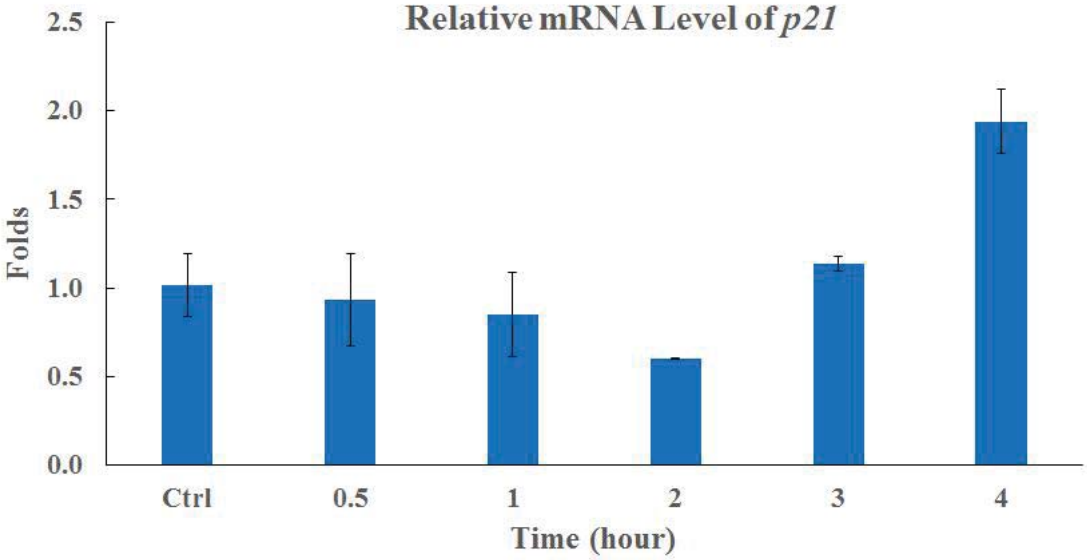
1
2
3
4
Time (hour)

c.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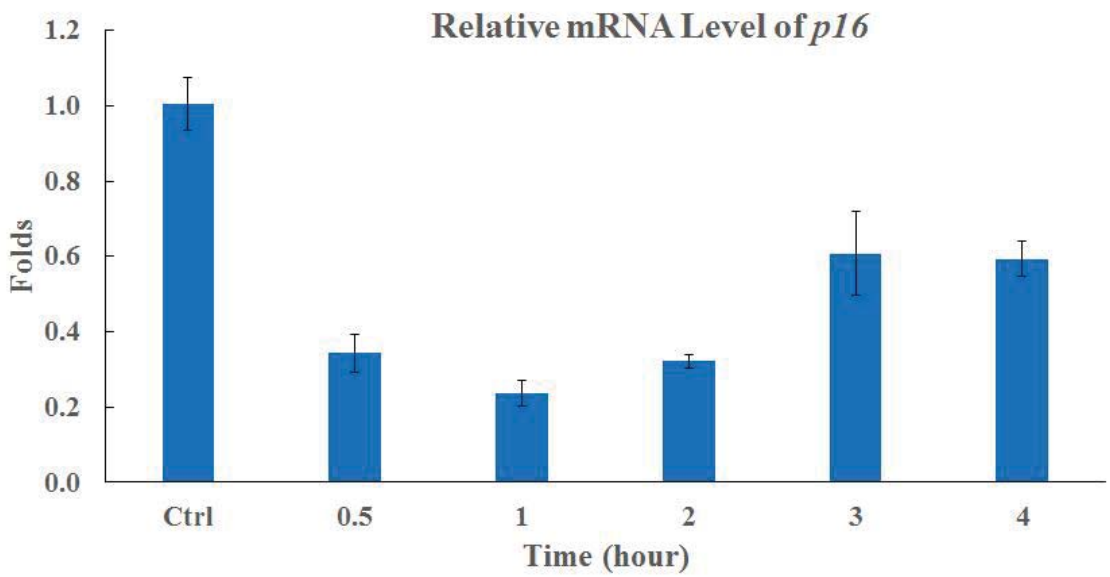
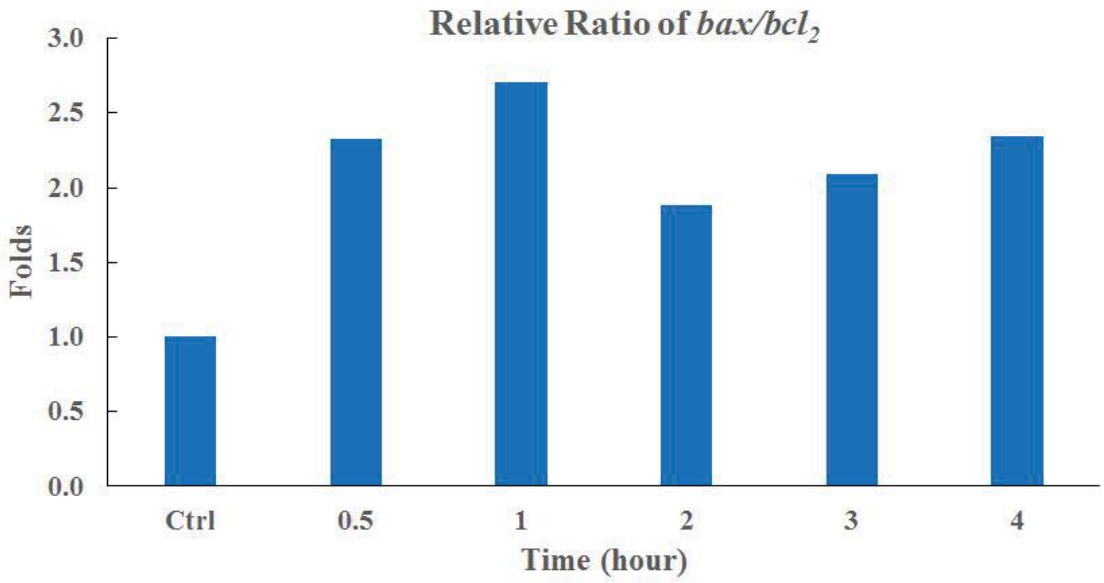
1
2
3
4
Time (hour)

d.


Relative mRNA Level of p16
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Folds
Ctrl
0.5
1
2
3
4
Time (hour)

e.


Relative Ratio of bax/bcl2
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Folds
Ctrl
0.5
1
2
3
4
Time (hour)

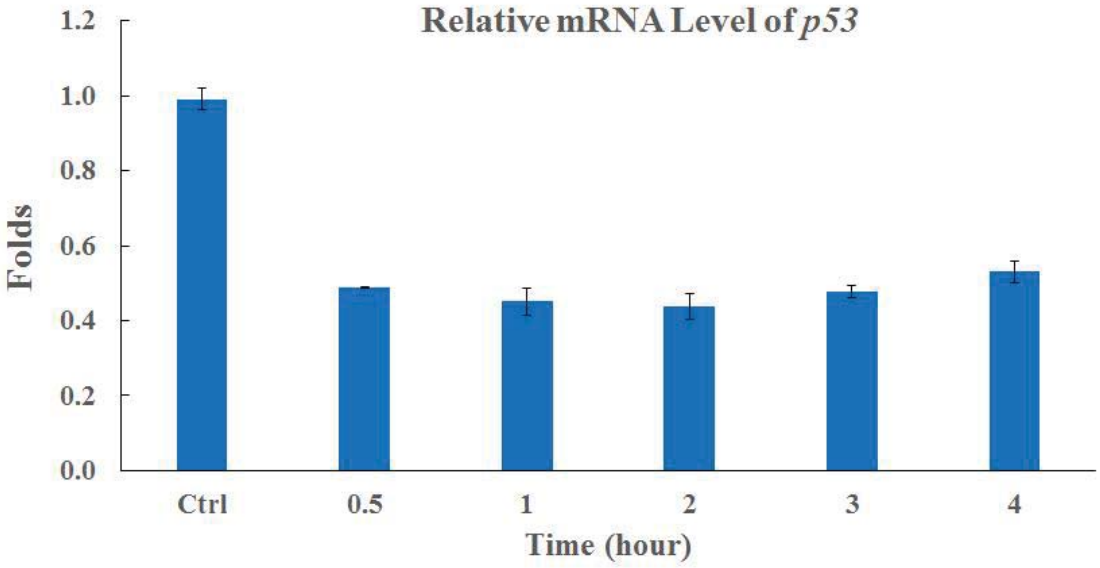
3. mRNA levels of genes involved in cycle arrest genes lowered, while mRNA levels of apoptotic genes increased after UV irradiation in wild-type BRAF and P53 human melanoma cell line (WM3918 cell line).

WM3918 is a metastatic melanoma cell line containing wild type *braf* and wild type *p53*. The gene expression at mRNA level for *p53* became 50% lower in response to UV light (Figure 3a). Also, the relative mRNA level of *mdm2* decreased by 50% and remained at a relatively constant level (Figure 3b). For the *p21* gene, the transcription level first decreased about 40% and then began to increase and finally reached a level that is much higher than that in the control group (Figure 3c). The *p16* gene in WM3918 was lost via homologous recombination, thus the mRNA level of *p16* in WM3918 after UV irradiation was barely detected. The relative ratio of *bax*/*bcl*$_2$ initially increased and then decreased at the time point of 2-hours after UV exposure (Figure 3d).

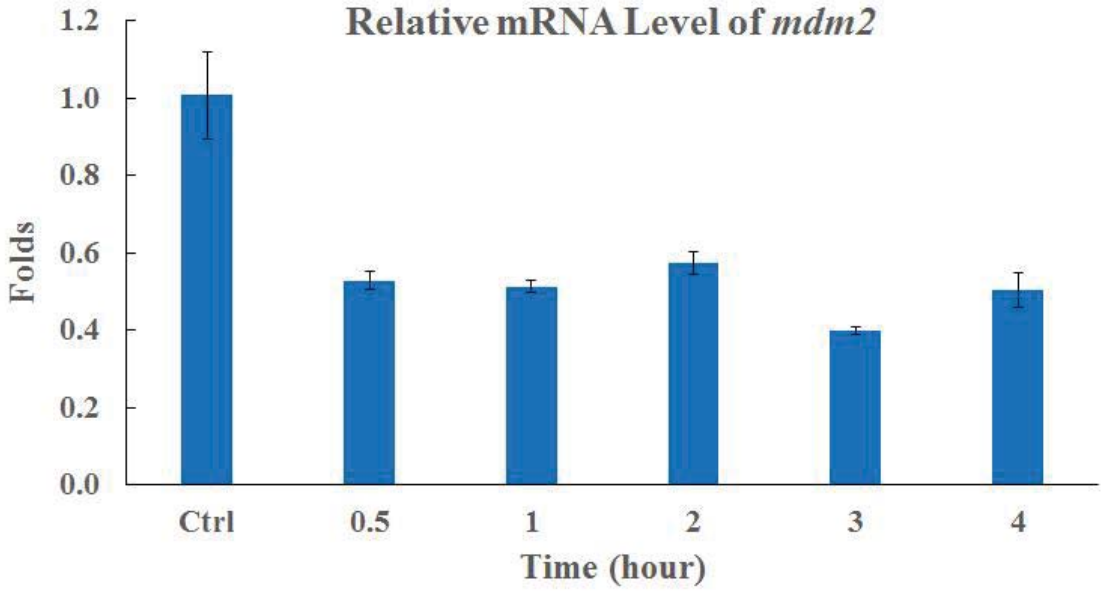
Figure 3. Relative mRNA levels of *p53* and P53 downstream genes in WM3918.

After UV irradiation, relative mRNA levels of *p53* (3a), *mdm2* (3b), *p21* (3c), and the ratio of *bax*/*bcl*$_2$ (3d) in WM3918 were measured by real-time qPCR. Each experiment was repeated two times with quadruplicate reactions for each gene. The results are represented as mean±SD.

a.


Relative mRNA Level of p53
Folds
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

b.


Relative mRNA Level of *mdm2*
Folds
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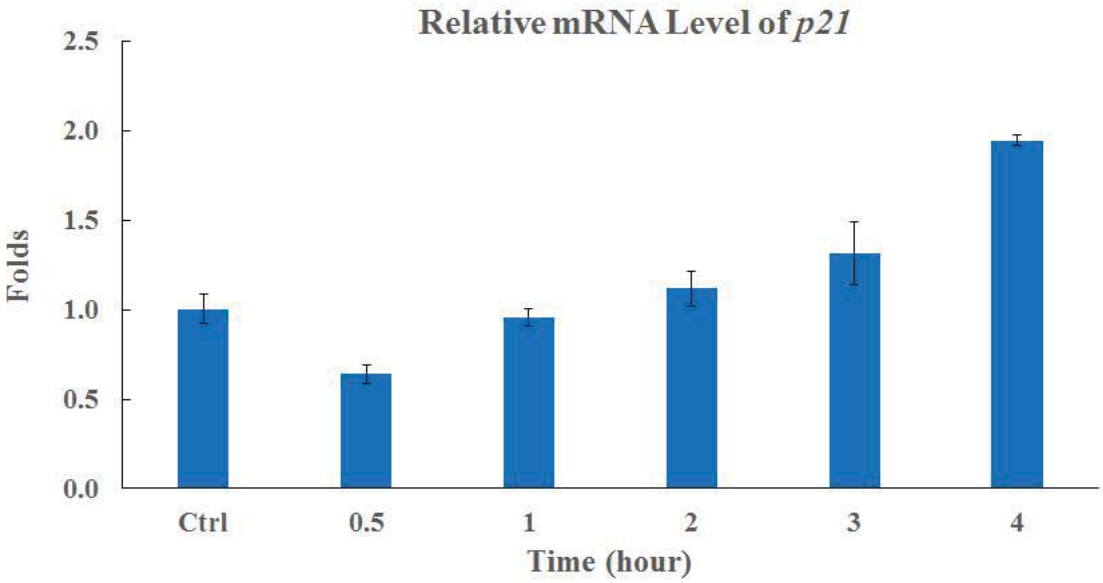
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

c.


Relative mRNA Level of p21
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2.0
1.5
1.0
0.5
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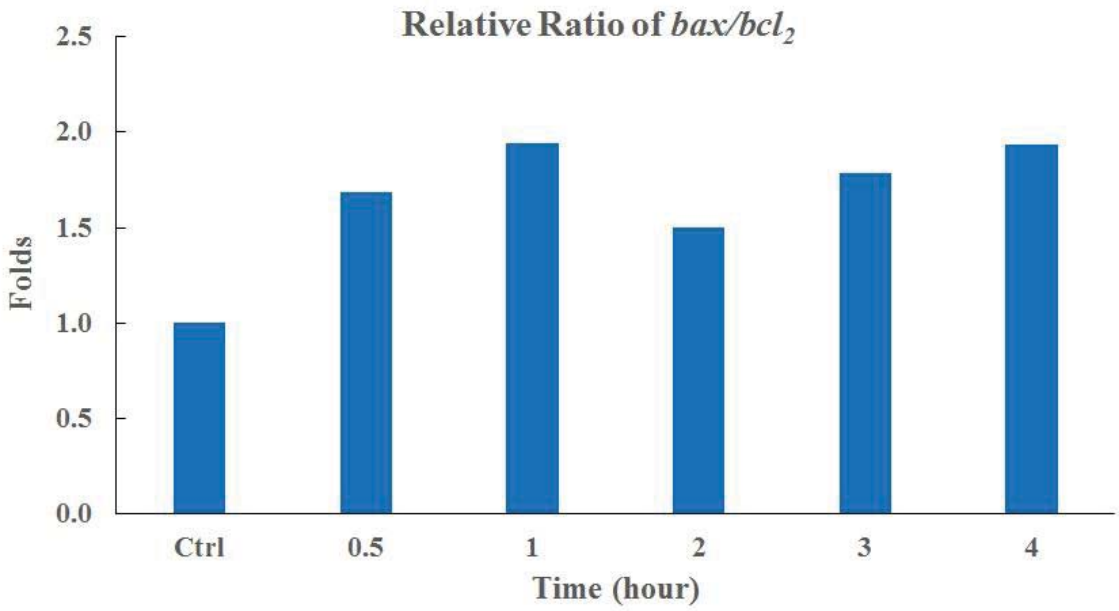
Folds
Ctrl
0.5
1
2
3
4
Time (hour)

d.


Relative Ratio of bax/bcl2
Folds
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

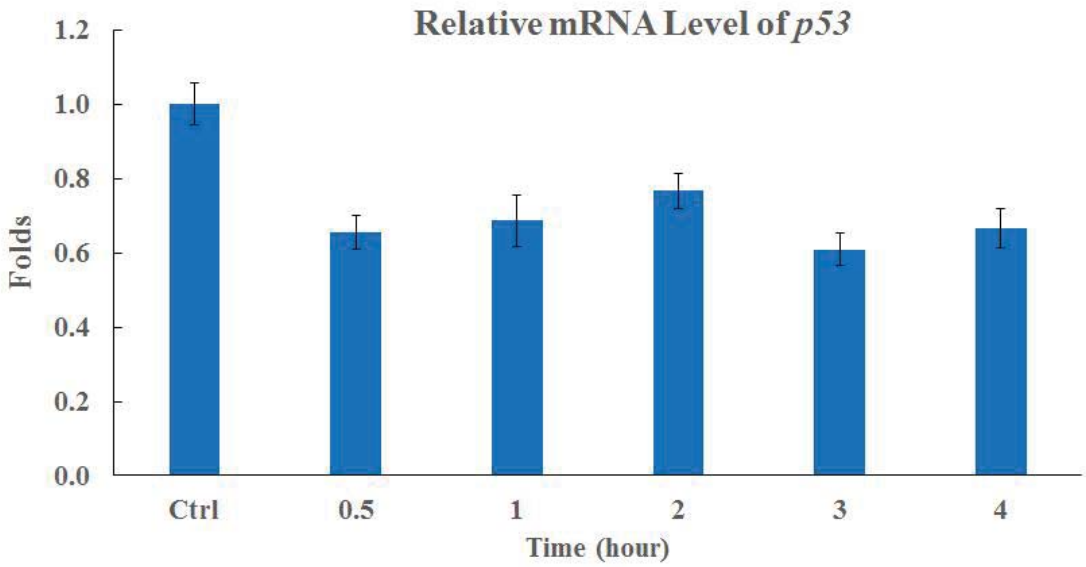
4. mRNA levels of genes involved in cycle arrest declined, while mRNA levels of apoptotic genes were elevated after UV irradiation in wild type BRAF and mutant P53 human melanoma cell line (WM3211 cell line).

The genetic context of the vertical-growth-phased malignant melanoma cell line WM3211 is wild type BRAF and $P53^{C242G}$. Similar to other melanoma cell lines, in response to UV exposure, the expression at the mRNA level of *p53* dropped and fluctuated a little (Figure 4a). For the transcription level of *mdm2*, after the UV light treatment, it showed a slow decrease and finally attained about a 50% reduction after 4-hour incubation (Figure 4b). The gene expression level of *p21* manifested a "wavy" tendency after UV irradiation, which first decreased and then climbed above the level of the control group and then declined after 3-hour incubation (Figure 4c). The expression level of *p16* plunged after the UV exposure and the fluctuations are shown after 1-hour incubation (Figure 4d). For the genes involved in apoptosis, gene expression levels of *bax* and *bcl2* both increased, and the relative ratio of *bax*/*bcl2* went up at the time point of 0.5-hours, then dropped to a level slightly higher than the control group and maintained at a relatively steady status (Figure 4e).

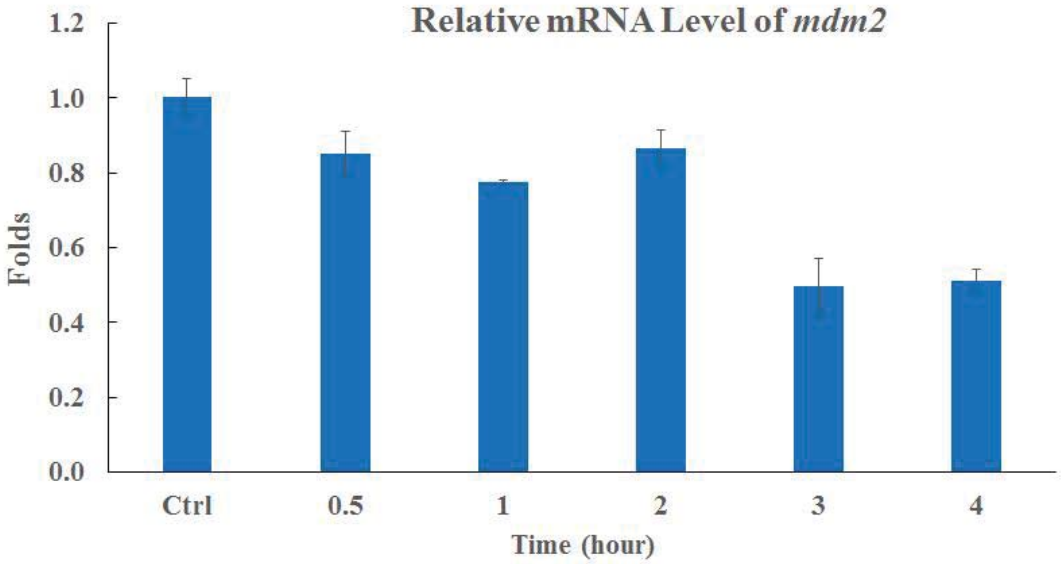
Figure 4. Relative mRNA levels of *p53* and P53 downstream genes in WM3211.

After UV irradiation, relative mRNA levels of *p53* (4a), *mdm2* (4b), *p21* (4c), *p16* (4d), and the ratio of *bax*/*bcl*$_2$ (4e) in WM3211 were measured by real-time qPCR. Each experiment was repeated two times with quadruplicate reactions for each gene. The results are represented as mean±SD.

a.


Relative mRNA Level of p53
Folds
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

b.


Relative mRNA Level of *mdm2*
Folds
1.2
1.0
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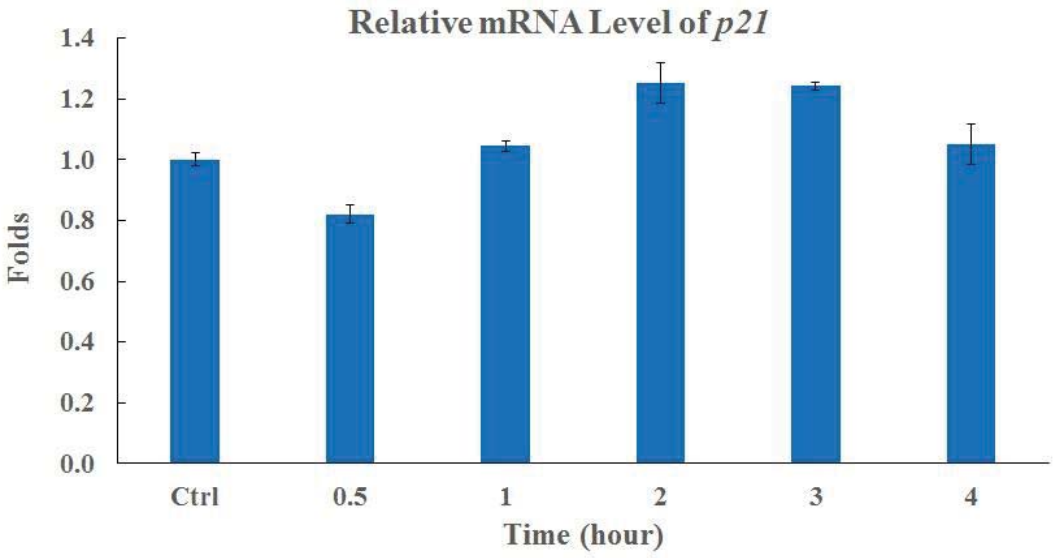
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

c.


Relative mRNA Level of p21
Folds
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1.2
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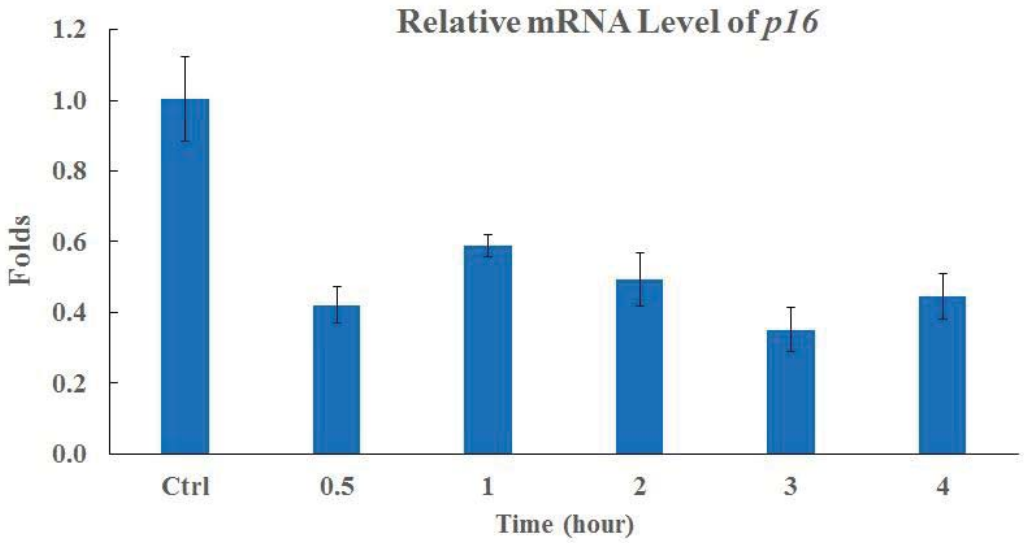
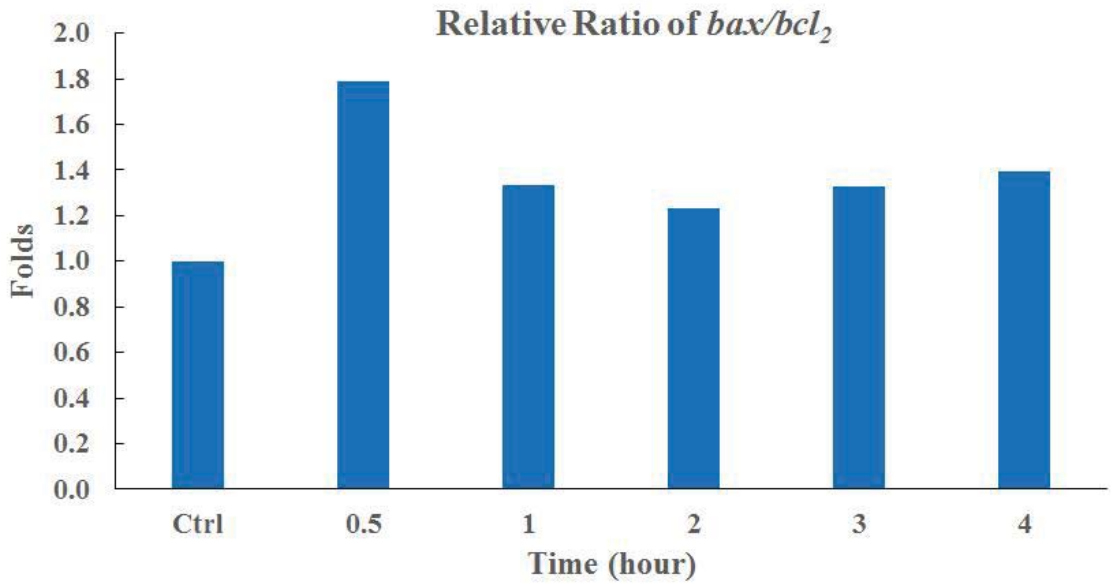
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

d.


Relative mRNA Level of p16
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Folds
Ctrl
0.5
1
2
3
4
Time (hour)

e.


Relative Ratio of bax/bcl2
Folds
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

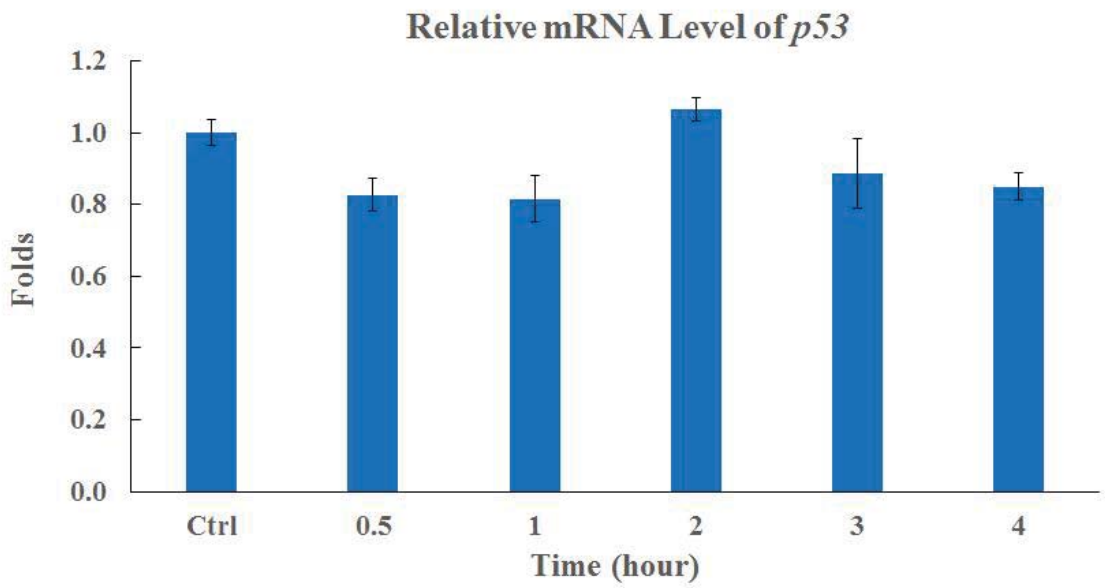
5. mRNA levels of genes involved in cycle arrest genes upregulated, while mRNA levels of apoptotic genes were dropped after UV irradiation in the normal melanocyte cell line.

Melanocyte is a type of melanin-producing normal skin cell. After UV irradiation, the expression at mRNA level of *p53* reduced mildly about 20% and recovered 2 hours after UV irradiation (Figure 5a). The transcription level of *mdm2* dropped sharply in response to UVR exposure (Figure 5b). For *p21*, the gene expression level first decreased and then increased after 1-hour incubation (Figure 5c). Also involved in cell cycle arrest, the UV treatment upregulated the gene expression at mRNA level of *p16*, although a decrease was observed after 2-hours incubation (Figure 5d). In regard to genes that regulate apoptosis, the transcription level of *bax* became lower and the level of bcl_2 rose, resulting in a reduced ratio of bax/bcl_2 (Figure 5e).

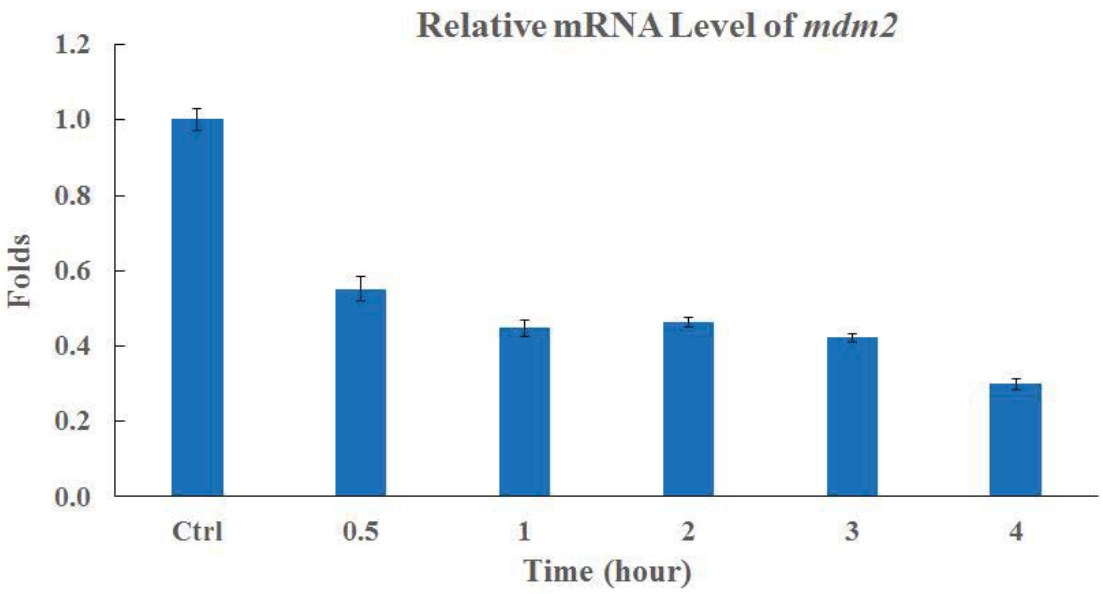
Figure 5. Relative mRNA levels of *p53* and P53 downstream genes in Melanocyte.

After UV irradiation, relative mRNA levels of *p53* (5a), *mdm2* (5b), *p21* (5c), *p16* (5d), and the ratio of *bax*/*bcl*$_2$ (5e) in melanocytes were measured by real-time qPCR. Each experiment was repeated two times with quadruplicate reactions for each gene. The results are represented as mean±SD.

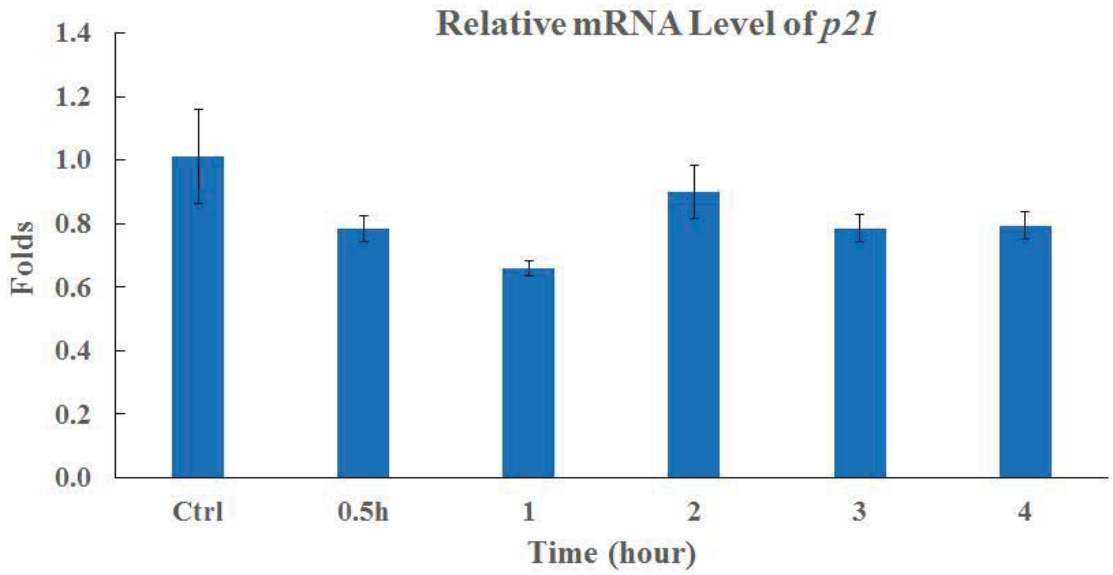
a.


Relative mRNA Level of p53
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Folds
Ctrl
0.5
1
2
3
4
Time (hour)

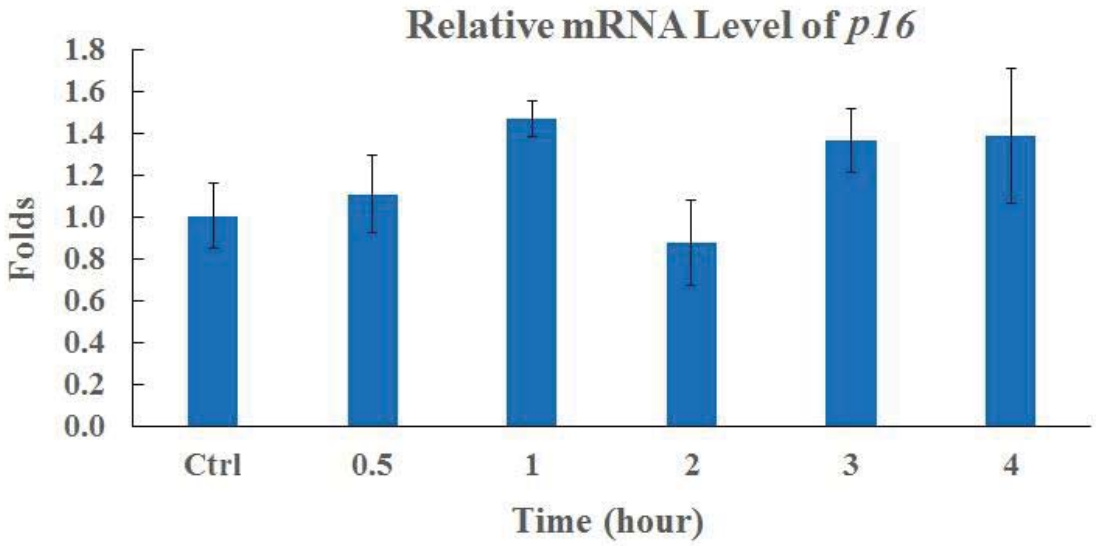
b.


Relative mRNA Level of *mdm2*
Folds
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

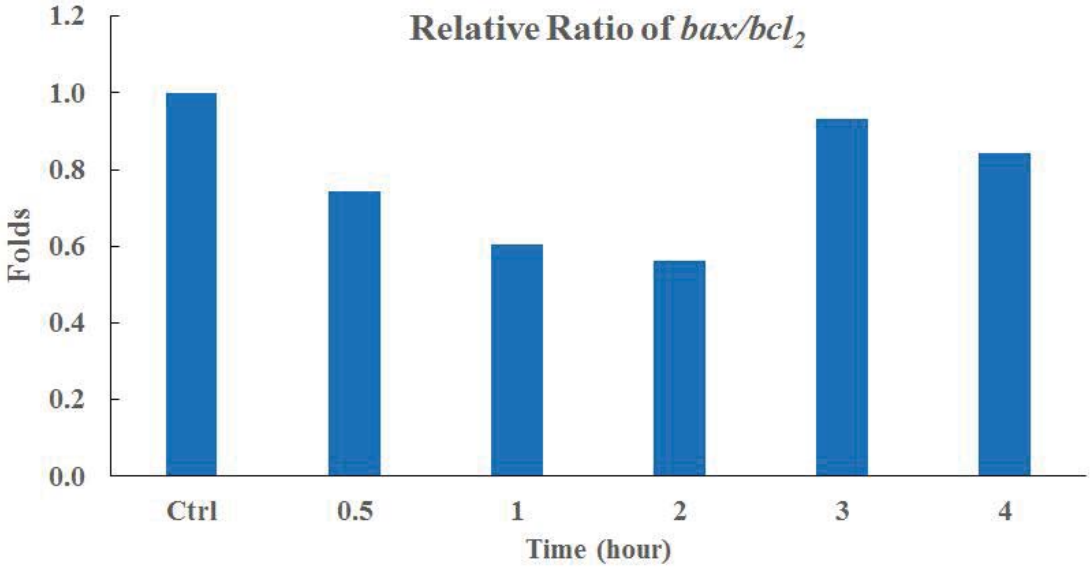
c.


Relative mRNA Level of p21
Folds
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5h
1
2
3
4
Time (hour)

d.


Relative mRNA Level of p16
Folds
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

e.

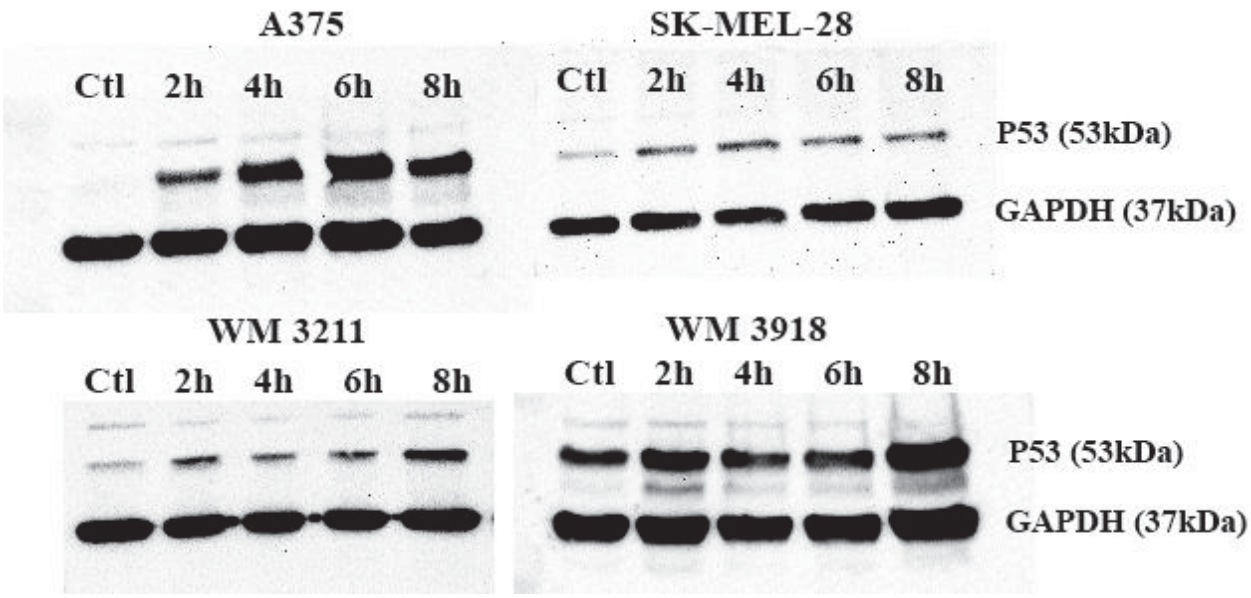

Relative Ratio of bax/bcl2
Folds
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctrl
0.5
1
2
3
4
Time (hour)

6. After UV irradiation, the P53 protein levels all increased in the four malignant melanoma cell lines.

After UV irradiation, the expression at the protein level of P53 in all the four malignant melanoma cell lines increased, but with different magnitudes of increase (Figure 6). The analysis by ImageJ image processing software indicated that the P53 protein level kept rising in the A375 cell line (Figure 6) throughout the four time points. For the other three cell lines, the protein levels of P53 fluctuated above the protein level of the control group. In SK-MEL-28, the protein level of P53 first increased and then decreased (Figure 6). In WM3918, the protein level of P53 had two peaks at the time points of 2-hours and 8-hours (Figure 6). Similarly, the total amount of P53 protein in the WM3211 cells was elevated in response to UV light and displayed two peaks at the time points of 2-hours and 8-hours (Figure 6).

Figure 6. UVC radiation increased P53 expression.

Western-blot analysis was conducted of P53 in A375, SK-MEL-28, WM3211, and WM3918 cells. GAPDH was used as the internal loading control. This experiment was repeated three times and a representative figure is shown.


A375
Ctl 2h 4h 6h 8h
SK-MEL-28
Ctl 2h 4h 6h 8h
P53 (53kDa)
GAPDH (37kDa)
WM 3211
Ctl 2h 4h 6h 8h
WM 3918
Ctl 2h 4h 6h 8h
P53 (53kDa)
GAPDH (37kDa)

Discussion

Melanoma is a mortal type of cutaneous malignant cancer with a poor prognosis [28]. The incidence rate of metastasized melanoma in the United States has increased approximately 4% to 6% annually [55]. Based on the estimated annual incidence, the lifetime risk of developing a melanoma is reported to be 1 in 54 [56]. Median survival after the occurrence of distant metastases of melanoma is only 6-9 months, and the 5-year survival rate is less than 5% [57]. Therapeutic options for malignant melanoma are dependent upon the stage at diagnosis. Surgery excision is the main treatment for the early stage of melanoma [58]. However, no effective therapy is currently available to cure melanoma in advanced stages. Therefore, there is an urgent need of developing effective therapeutic strategies that improve prevention and treatment of metastatic melanoma.

p53, a master guardian of human cells, is in charge of initiating processes such as cell cycle arrest, programmed cell death, and DNA damage repair in response to physiological stress signals, thereby ensuring that cells keep their household in order. In many types of cancer, the P53 pathway is usually defective because of the genetic mutations occurring within the *p53* sequence [59]. The missense mutations of *p53* can be divided into two subgroups: one category occurs in the DNA-contact sequence on P53 (e.g. R248) which disrupts the interaction of P53 with the promoters of its downstream genes; another group includes mutations happening on the essential residues critical for functional P53 conformation (e.g. R175), subsequently altering the three-dimensional structure of the protein [60]. The mutant P53 exerts its activity in either a dominant-

negative manner in which it interacts with wild-type P53 and interrupts the normal transactivation of P53 [14], or via a gain-of-function mechanism [60] in which mutant P53 manifests novel activity supporting tumor progression [60, 61].

UVR accelerates melanogenesis [29, 62], and it activates the P53 pathway in the melanoma as well. There are three types of ultraviolet radiation based on wavelengths: UVC (180-280 nm), UVB (280-320 nm) and UVA (320-400 nm). Since several kinases show a differential wavelength-dependent response to UV light, the response of P53 and its effectors to UV light is dependent on the wavelength of UV light [24]. In this experiment, UVC, whose energy is the highest, was chosen. Binding of the UV photoproducts to ATR induces its kinase activity after UVC irradiation, which phosphorylates P53 and regulates expression of genes in control of P53 [63].

In normal cells, because the onco-protein MDM2 conceals the activation domain of P53 and mediates the ubiquitin-protease pathway to degrade P53 [64], P53 maintains its expression at a low level. When P53 is modified by phosphorylation, the accumulation of P53 can activate expression of *mdm2*, which encodes the MDM2 protein and negatively regulate the P53 protein level. Usually, a high protein level of P53 is correlated to an increased mRNA level of *mdm2*.

Our results indicate the existence of defects in the capacity of P53 to target the genes *mdm2*, *p21*, *bax* and *bcl2* in UV-challenged melanoma cell lines. It was surprising that in all of the four melanoma cell lines, the mRNA level of *p53* decreased after the

UVC exposure, despite the gene contexts of the *p53* in different melanoma cell lines. However, the western blotting results showed that P53 protein levels in all melanoma cell lines was upregulated, instead of decreased, which is consistent with the results in Pedeux‘s experiment [65]. However, the gene expression level of *p53* in melanocytes only slightly declined. Although we have no data demonstrating the protein level change in melanocyte cells, we can predict that the protein level will increase after UVR exposure. In regard to the expression levels of *mdm2*, after UV radiation, the decreasing expression level of *mdm2* explains the increasing P53 protein level because the inhibitory effect of MDM2 was restrained. In terms of P53 protein levels, even though it is in the cells with wild type P53, it fails to upregulate the mRNA level of *mdm2*, indicating that other pathways are involved in downregulating *mdm2*. Moreover, *mdm2* was downregulated in normal melanocytes, which might indicate that after UV irradiation, it is a feature that skin cells producing melanin have a decreased expression of *mdm2*.

p21$^{Waf1/Cip1}$ is a P53-regulated gene that encodes a cyclin-dependent kinase inhibitor whose activation is essential for P53-mediated G1 cell cycle arrest [66]. It was also found that P21 participates in both P53-dependent and P53-independent programmed cell death after physiological stress [67]. The presence of P21$^{Waf1/Cip1}$ protects melanoma cells from apoptosis, conferring a survival advantage [67]. Moreover, P21 plays a central role in the determination of cell fate. When DNA is mildly damaged, P53 accumulates to moderate levels and *p21* is induced to arrest the cell cycle, allowing for DNA repair. For severe DNA damage, P53 is driven to high levels, leading to a decreasing level of *p21* and induction of apoptotic genes including *bax* [68].

In our experiments, after the irradiation of UVC, expression at mRNA level of *p21* always decreased, which is different from the results of Pedeux's and Haapajarvi's experiments [53, 65]. In addition, the relative ratios of the gene expression of *bax*/*bcl*$_2$ were all elevated. These results indicate that a reduced level of *p21* in the melanoma cells promote the apoptosis of melanoma cells in response to UV irradiation, and the upregulated pro-apoptotic gene levels are associated with low cell survival of melanomas. Instead of inducing cell cycle arrest after UV irradiation, cells are prone to induce apoptosis. According to the relative ratio of *bax*/*bcl2*, melanoma cells with wild type P53 have similar level of apoptosis induction, while melanoma cells with mutant P53 displayed either higher or lower induction. The difference in the induction between two cell lines with mutated P53 might be attributed to the positions of missense mutations. The P53 mutation of SK-MEL-28 occurs on 145, belonging to the conformational mutation, whereas the P53 mutation of WM3211 happens on 242, interrupting the affinity of P53 binding to DNA.

In the two melanoma cell lines with wild type P53, P53-target-genes behaved similarly, while in the two melanoma cell lines with mutated P53, the subtle difference of the gene expression might result from the mutant type of P53. Despite this subtle difference, all of the melanoma cell lines exhibit similar patterns in the activation of genes in the P53 network in response to UV irradiation. There could be two contemplative hypotheses to explain this phenomenon. 1) The UVC radiation dosage is beyond the range of DNA repair and other adaptive abilities of melanoma cells and thus

induces irreversible gene mutations and other genetic instabilities that are either fatal or able to deprive melanoma cells of the advantages in cell proliferation and metastasis. As a result, they undergo apoptosis to allow other melanoma cells to grow, which can be considered a socially responsible behavior for the whole tumor mass. 2) The P53 network is severely deregulated. Other mechanisms or pathways are activated to compensate the regulation of the severely deregulated P53 network in responding to UV damages. Therefore, melanoma cells respond to UV irradiation in a similar way despite the gene context of *p53* or *braf*.

P16, a cyclin-dependent kinase-4 inhibitor (CDK4) similar to P21, has been found to be associated with several human cancers, especially some familial melanomas [69, 70]. P16 is involved in inducing G1 and G2 cell cycle arrest and inhibiting apoptosis in response to UV light [71]. *p16* downregulation was observed in our results, suggesting the induction of apoptosis in the melanoma cell lines. The expression at the mRNA level of p16, however, was increased in the A375 cell line, which contains mutations in $p16^{CDKN2A}$ gene.

In the normal skin cell melanocyte, the increase in mRNA of *p16* occurred earlier than when the ratio of bax/bcl_2 started to decrease, suggesting that the cells prefer to stop cell cycle, allowing DNA repair instead of inducing apoptosis. It is reversed in melanoma cells.

Future experiments might focus on the other pathways that might be involved in the responses to UV-induced apoptosis and cell cycle; among them, *p16* might be a suitable choice [72]. More melanoma cell lines with more gene contexts, such as *pten*+/+ or *pten*-/- and *p16*+/+ or *p16*-/-, are needed to evaluate and strengthen our findings. In order to evaluate our hypothesis about how the p53 network is deregulated in melanoma cells, other UV dosage, either higher or lower than $50J/m^2$, can be used to examine if there will be a different response of the P53 network in reacting to UV irradiation. Also, since the alteration of expression at the mRNA level is not a direct indicator of the protein level change due to post-transcriptional and post-translational modifications, the protein level of all of the P53 target genes needs to be analyzed.

Understanding the abnormal behaviors of genes in P53 network can lead us to identify the genes or other crucial pathways in the P53 controlling network that we can target to restore the regulation and function of P53 network to maintain the integrity of the cells.

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