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ORIGINAL RESEARCH

Silencing *TCF4* Sensitizes Melanoma Cells to Vemurafenib Through Inhibiting *GLUT3*-Mediated Glycolysis

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Background: Vemurafenib is a selective BRAF inhibitor with significant early effects in melanoma, but resistance will develop with the duration of treatment. Therefore, overcoming vemurafenib resistance can effectively improve the survival rate of melanoma. The transcriptional activity of *TCF4* is necessary to maintain the malignant phenotype of cancer cells. However, the effect of *TCF4* on melanoma sensitivity to vemurafenib and the underlying mechanism is unclear.

Methods: Vemurafenib-resistant A375 (A375/Vem) and SK-Mel-28 (SK-Mel-28/Vem) cells were constructed by administering increasing concentrations of vemurafenib, and the expression of *TCF4* was examined in parent and vemurafenib-resistant cells. *TCF4* loss-function cells models were established in A375/Vem and SK-Mel-28/Vem cells, respectively. Cell survival, clone formation, and cell apoptosis were assessed. The downstream target gene of *TCF4* was verified by chromatin immunoprecipitation. Finally, the effect of *TCF4* on melanoma cells glycolysis was investigated and were performed.

Results: *TCF4* expression was increased in vemurafenib-resistant melanoma cells, and knocking down *TCF4* could promote the sensitivity of melanoma cells to vemurafenib. Mechanism investigation revealed that *TCF4* could interact with *GLUT3* and silencing *TCF4* could inhibit *GLUT3* expression. In addition, overexpression of *GLUT3* reversed the growth and glycolysis of tumor cells that were inhibited by *TCF4* knockdown.

Conclusion: Our study demonstrates that *TCF4* downregulation sensitizes melanoma cells to vemurafenib through inhibiting *GLUT3*-mediated glycolysis. These findings support *TCF4* as an oncogene and provide new mechanism by which *TCF4* confers chemotherapy resistance in melanoma.

Keywords: melanoma, chemotherapy resistance, BRAF inhibitor, glucose transporter, transcription factor 4, Warburg effect

Introduction

Melanoma is a common malignant tumor with an increasing incidence. There are approximately 132,000 new cases of melanoma each year worldwide.[1] Approximately 25% of patients with advanced melanoma have a one-year survival time, with an average survival time of only 6.2 months.[2] Melanoma is divided into four groups by genotype: BRAF mutation, NRAS mutation, NF-1 deletion, and wild type that have no mutations with these three genes.[3] About 50% of melanoma have a point mutation in the BRAF gene. The most common mutation is V600E, which mutates from valine to glutamic acid, activates the

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MAPK (RAS-RAF-MEK-ERK) signaling pathway, thereby promoting the continued growth of tumor cells.[4] At present, there are many marketed drugs for the treatment of melanoma, such as, BRAF inhibitors (vemurafenib, dabrafenib) and MAPK/MEK inhibitors (trametinib, cobimetinib), which significantly improve the prognosis of patients with advanced melanoma.[5] Vemurafenib is a selective BRAF inhibitor with significant early effects, but resistance will develop with the duration of treatment, especially after 6–8 months of treatment.[6] Therefore, overcoming Vemurafenib resistance can effectively improve the survival rate of melanoma.

Transcription Factor 4 (*TCF4*) is one of the major proteins in the Wnt/β-catenin pathway.[7] *TCF-4* is a member of the T cytokine/lymph enhancer (TCF/LEF) family. *TCF4* located on the chromosome 10q25.3 and coded by *TF7L2* (transcript factor 7 like 2) gene.[8] This gene contains 17 exons, has a nuclear localization signal domain (NLS), the exon 1 encoding the β-catenin binding region; exon 10–11 encoded high mobility histone domain (HMG-box) which can recognize specific DNA sequences.[8] In many tumors, abnormally activated Wnt pathway leads to nucleus translocation of β-catenin and combination with the relevant domains of *TCF4* to form a transcription complex, which promotes the overexpression of downstream target genes and promotes the occurrence and development of tumors.[9] Therefore, the transcriptional activity of *TCF4* is necessary to maintain the malignant phenotype of cancer cells. Previous studies have shown that inhibiting the binding of *β-catenin* /*TCF4* can promote the radiosensitivity of lung cancer cells,[10] and other studies have found that *TCF4* can mediate neuroendocrine differentiation and result in the resistance of prostate cancer cells to enzalutamide,[11] indicating that *TCF4* is associated with drug resistance in cancer cells. In melanoma, *TCF4* can be inactivated by *PGC1α*, which is a transcriptional coactivator that promotes mitochondrial biogenesis, protects against oxidative stress and reprograms melanoma metabolism to influence drug sensitivity and survival.[12] Inactive *TCF4* causes downregulation of metastasis-related genes. In addition, vemurafenib treatment suppresses metastasis by acting on the *PGC1α*/*TCF4* axis.[12] However, the role of *TCF4* in vemurafenib-resistant melanoma remains unknown.

In this study, we knocked down *TCF4* in vemurafenib-resistant melanoma cells and examined its effects on melanoma sensitivity to vemurafenib, evaluated by cell colony and cell apoptosis. The underlying mechanism was also explored.

Materials and Methods

Cell Culture and Generation Vemurafenib-Resistant Cells

Melanoma cell lines, A375 and SK-Mel-28, were purchased from National Infrastructure of Cell Line Resource (Beijing, China). Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (GlutaMAX™-I, cat no. 72400120, Gibco). Ten percent fetal bovine serum (Gibco, CA, USA) was added in the medium. Cells were cultured under the condition in a 37° C humidified atmosphere of 5% CO_2. The vemurafenib-resistant A375 and SK-Mel-28 cell lines (A375/Vem and SK-Mel-28/Vem) were established in our lab. A375 and SK-Mel-28 cells (1 × 105/mL) were treated with the sequential increases of vemurafenib concentrations, from 0.5 to 6.0 μM every 3 days for 6 weeks. Then, cell colonies were isolated.[13] A375/Vem and SK-Mel-28/Vem cells were, respectively, replenished with 1.0 or 2 μM vemurafenib every 3 days.

Cell Transfection

SiRNAs were ordered from GenePharm (Shanghai, China). siRNA knockdown was carried out with two siRNAs targeting the *TCF4* cDNA sequence. The sequences as following: *TCF4* siRNA-1: 5′-AGAGAAGAGCAAGCGAAA UAC-3′, siRNA-2: 5′- UAGCUGAGUGCACGUUGAA AG-3′. Scramble oligonucleotides were used as a negative control. TCF4 cDNA ORF plasmid was purchased from Sino Biological (Beijing, China). Cells were transfected by Lipofectamine 2000 (Thermo Fisher, USA) following the manufacturers' instructions. Cells were harvested at 48 h after transfection.

CCK-8 Assay

Cell proliferation rates were measured using Cell Counting Kit-8 (CCK-8) (Beyotime, Hangzhou, China). A total of 0.5×10^4 cells were seeded in each 96-well plate for 24 h. After treatment, the cells were further incubated for 24 h. Ten microliter CCK-8 reagents were added to each well at 1 h before the endpoint of incubation. OD 570 nm value in each well was determined by a microplate reader.

Immunoblotting

After indicated treatment, the cells were lysed by RIPA lysis buffer (Cell Signaling Technology) for 30 min on ice. The 50 g protein sample was subjected to 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked by 5% non-fat milk for 1 h at room temperature. The membranes then were incubated with primary antibodies (anti-MDR, cat no. sc-55510, 1:1000, Santa Cruz Biotechnology; anti-P-gp, cat no. ab103477, 1:1000, Abcam; anti-TCF4, cat no. sc-166699, 1:1000, Santa Cruz Biotechnology; anti-GLUT3, cat no. sc-74399, 1:1000, Santa Cruz Biotechnology; and anti-β-actin, cat no. sc-47778, 1:1000, Santa Cruz Biotechnology) overnight at 4°C. The membranes were washed three times with PBS. After washing, the membranes were incubated with appropriate secondary antibodies for 1 h at 37°C. The bands were visualized by enhanced chemiluminescence.

Apoptosis Analysis

Annexin V-FITC Apoptosis Detection Kit (cat no. CA1020-100T, Solarbio, Beijing, China) was used in this experiment. After treatment, cells were collected and then resuspended in binding buffer. Annexin V-FITC (5 μL) and Propidium Iodide (5 μL) were added and mixed well. The cells were incubated with the mixed solution for 15 min. Finally, flow cytometric analysis was performed in Attune NxT (Thermofisher, Shanghai, China).

Clone Formation Assay

After treatment, the cells were collected and resuspended in the medium at 2000 cells per mL, and then were seeded into a 60 mm culture dish at 1000/dish. The cells were cultured for 1–2 weeks in a 37°C 5% CO2 incubator. When macroscopic clones appeared in the culture dish, the culture was terminated. The supernatant was discarded and washed 3 times with PBS. The cells were fixed with 1 mL of 4% paraformaldehyde for

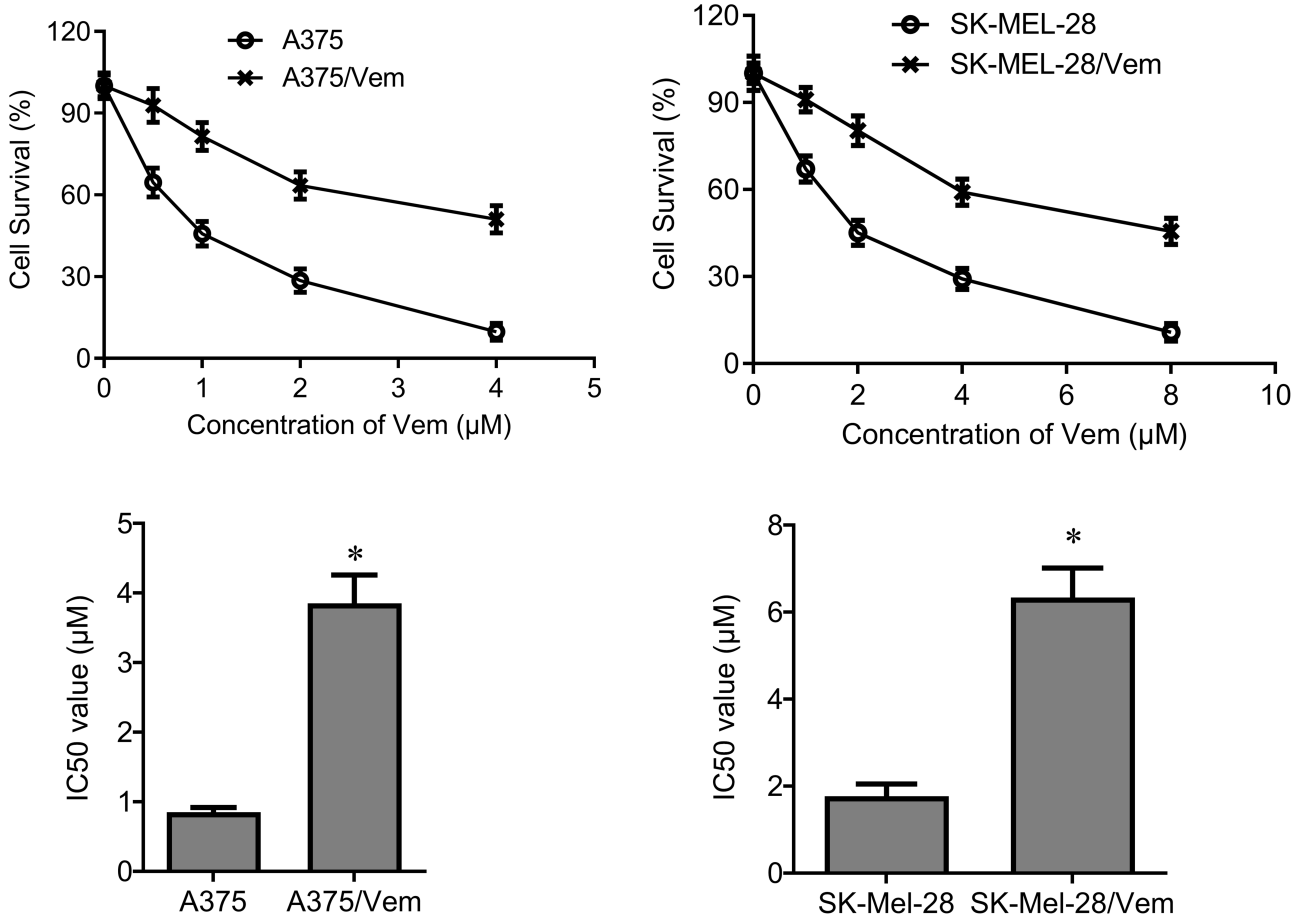

Figure 1 IC50 in Vemurafenib-resistant A375 and SK-Mel-28 cells. Vemurafenib-resistant A375 (A375/Vem) and SK-Mel-28 (SK-Mel-28/Vem) cell lines were established. The cells were treated with a series of concentrations of Vemurafenib (Vem). CCK-8 assay was performed to measure the cell viability in A375/Vem and SK-Mel-28/Vem cells after 48-h treatment. IC50 was calculated for A375/Vem and SK-Mel-28/Vem cells and their parental cells. Each experiment was performed three times. *p<0.05.

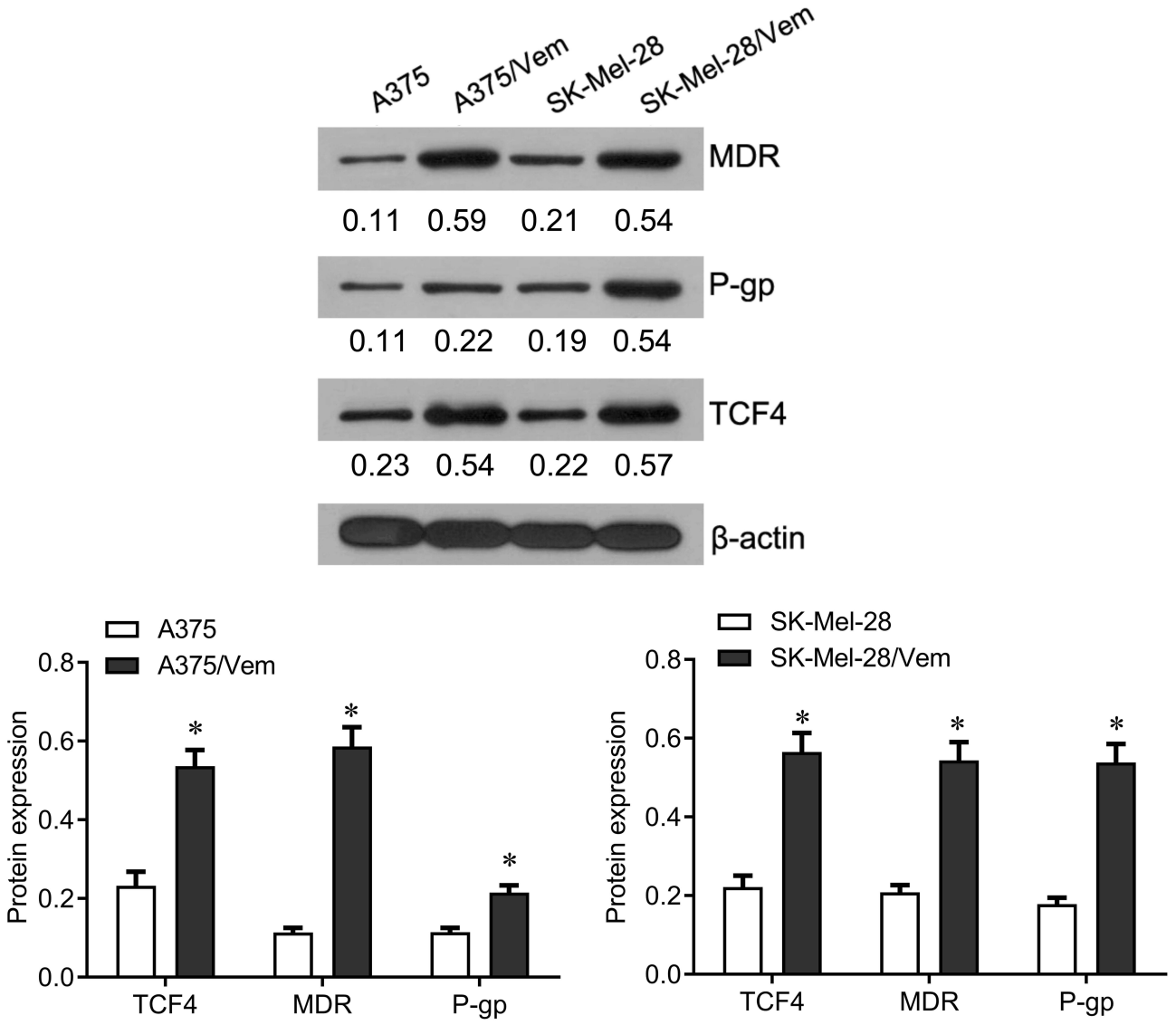

Figure 2 The expression of *TCF4* in Vemurafenib-resistant A375 and SK-Mel-28 cells. Western blot was performed to examine the expression of *TCF4* and the markers of drug resistance, MDR and P-gp in Vemurafenib-resistant A375 and SK-Mel-28 cells and their parental cells. Each experiment was performed three times. Vem, vemurafenib. *p<0.05.

15 min and then incubated with crystal violet for 20 min. After washing with PBS for 3 times, the clones were counted directly in a microscope (low magnification). Finally, the clone formation rate was calculated as follows: (number of clones/number of cells inoculated) × 100%.

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) assay was performed using the EZ-Magna ChIP kit (EMD Millipore). Cells were fixed with 4% paraformaldehyde and incubated with glycine for 10 min to generate DNA–protein cross-links. Cells were lysed with Cell Lysis Buffer and Nuclear Lysis Buffer, and sonicated to generate chromatin fragments of 400–800 bp. The lysates were immunoprecipitated with Magnetic Protein A Beads conjugated with *TCF4* antibody (rabbit monoclonal; Merck, Millipore) or rabbit non-immune IgG (as negative control). The precipitated DNA was analyzed by PCR. The following primers were used: *Glut3*-F: ATGCACATCCTGTATTATCC, *Glut3*-R: GAACAGACTGTTACAGTTGG.

Measurement of ATP

ATP Assay Kit (cat no. S0026B) was purchased from Beyontime Biotechnology (Shanghai, China) and used to detect the ATP concentrations in cells according to the manufacturer's instructions.

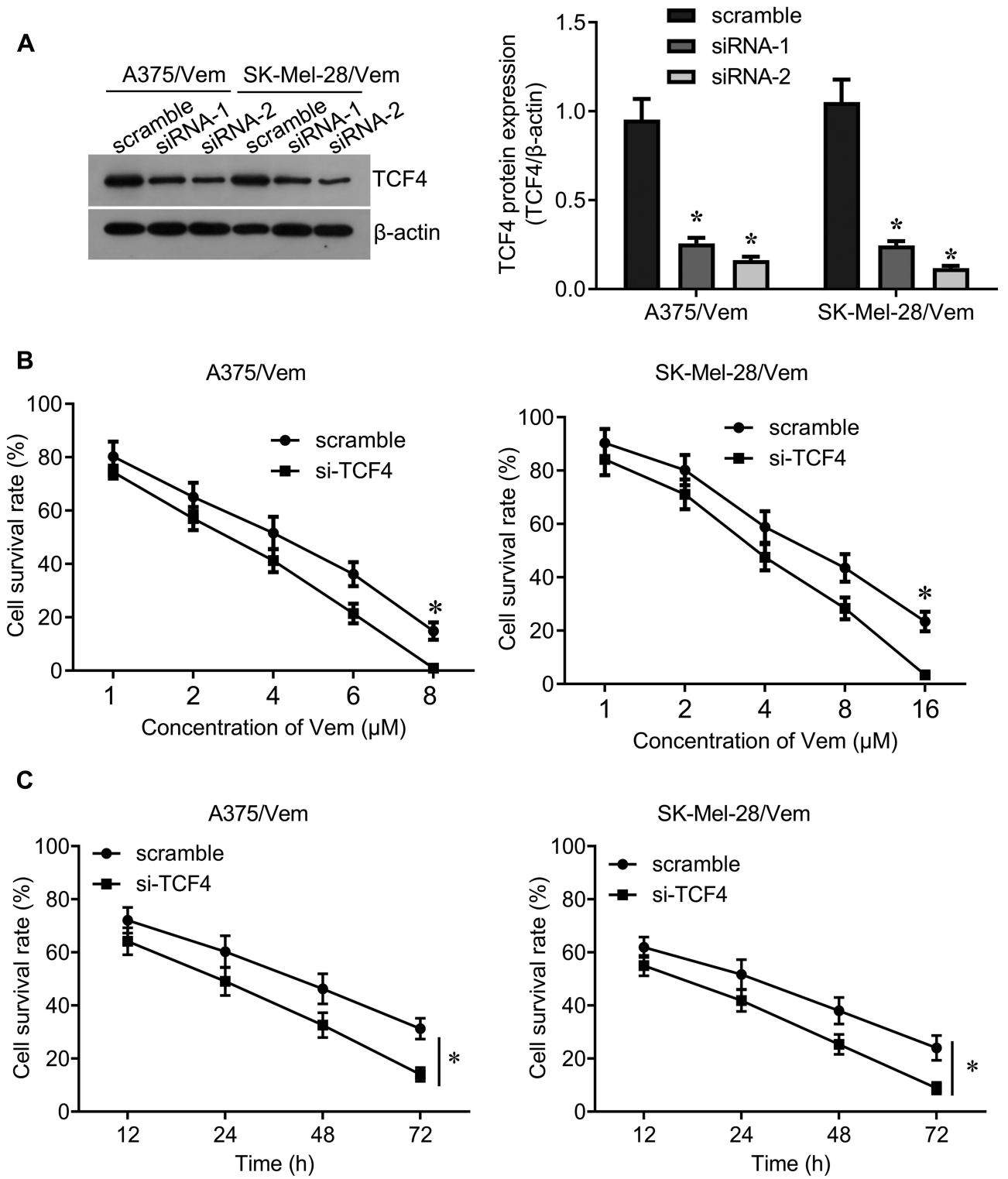

Figure 3 Silencing *TCF4* sensitizes A375 and SK-Mel-28 cells to Vemurafenib. (**A**) Western blot was performed to examine the expression of *TCF4* in Vemurafenib-resistant A375 and SK-Mel-28 cells after siRNA transfection (left), and quantification of the bands (right). (**B**) CCK-8 assay was performed to measure the cell viability in A375/Vem and SK-Mel-28/Vem cells after siRNA transfection and Vemurafenib treatment. (**C**) CCK-8 assay was performed to measure the cell viability at different times after siRNA transfection and vemurafenib treatment (A375/Vem, 3 μM; SK-Mel-28/Vem, 6 μM). Each experiment was performed three times. Vem, vemurafenib. *p<0.05.

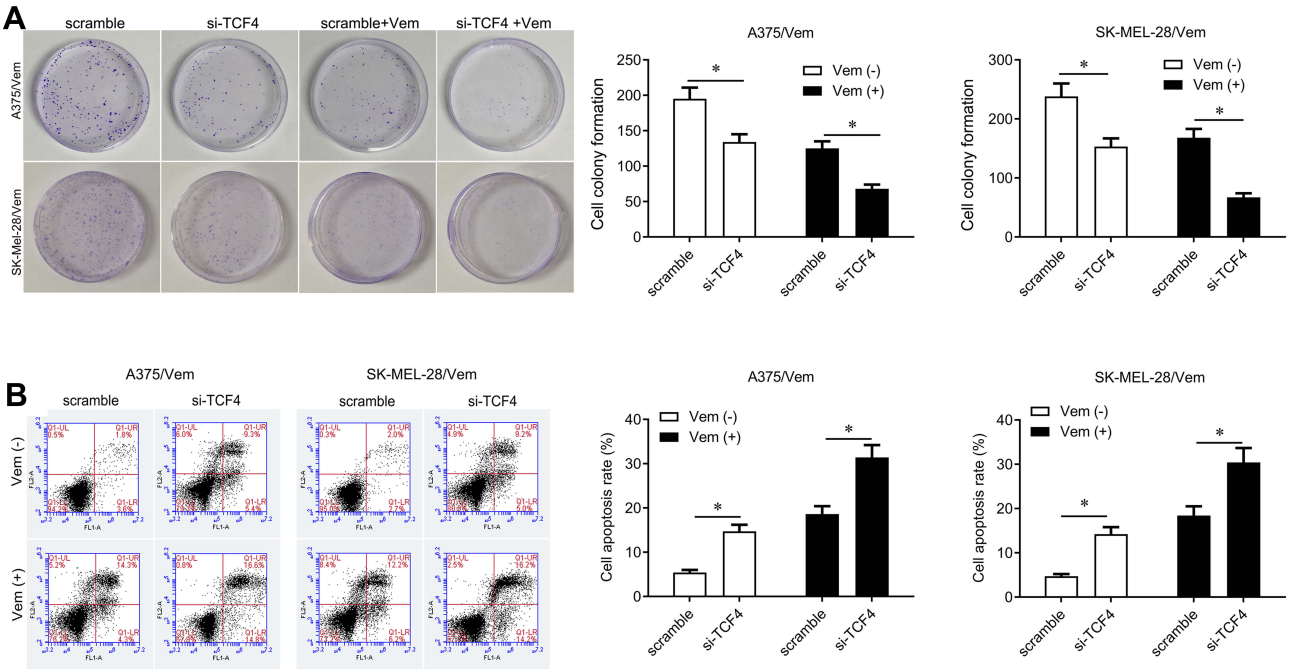

Figure 4 Silencing *TCF4* inhibits cell colony and promotes cell apoptosis in Vemurafenib-resistant A375 and SK-Mel-28 cells. (**A**) Colony formation was performed to measure the ability of cell proliferation in A375/Vem and SK-Mel-28/Vem cells after siRNA transfection and Vemurafenib treatment. (**B**) Cell apoptosis was analyzed to measure the apoptotic rate in A375/Vem and SK-Mel-28/Vem cells after siRNA transfection and Vemurafenib treatment. Each experiment was performed three times. Vem, vemurafenib. *p<0.05.

Measurement of Glucose Uptake

Glucose Uptake Assay Kit (cat no. KA4085) was purchased from Abnova (Taipei City, Taiwan) and used to detect the glucose uptake in cells according to the manufacturer's instructions.

Measurement of Lactate Level

D-Lactate Assay Kit (Colorimetric) (cat no. ab83429) was purchased from Abcam (Shanghai, China) and used to detect the lactate level in cells according to the manufacturer's instructions.

Statistical Analysis

GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses. Student's *t*-test for two group and one-way ANOVA with post hoc Bonferroni test for three or more group were used to assess the statistical significance. $P < 0.05$ was considered statistically significant.

Results

TCF4 Is Upregulated in Vemurafenib-Resistant Melanoma Cells

To investigate the role of *TCF4* in vemurafenib-resistant melanoma cells, we established the vemurafenib-resistant melanoma cells, A375/vem and SK-Mel-28/vem. The A375/vem and SK-Mel-28/vem and their parental cells were exposed to a series of concentration of vemurafenib. The cell viability was significantly decreased in all cells. However, the rates of descent in A375/vem and SK-Mel -28/vem cells were lower than that of in their parental cells (Figure 1). The IC50 of A375/vem (3.8 μM) and SK-Mel -28/vem (6.1 μM) were significantly higher than in A375 (0.8 μM) and SK-Mel-28 (1.8 μM) (Figure 1). In addition, the markers of drug resistance, MDR and P-gp, were significantly increased in A375/vem and SK-Mel-28/vem cells compared with their parental cells (Figure 2). We then tested the expression of *TCF4* by Western blot, the expression of *TCF4* was significantly upregulated in A375/vem and SK-Mel-28/vem cells compared with their parental cells (Figure 2), indicating an important role of TCF4 in vemurafenib resistance.

Downregulation of *TCF4* Sensitizes Melanoma Cells to Vemurafenib

We further investigated whether knocking down *TCF4* could sensitize melanoma cells to vemurafenib. We designed two siRNA sequences to knock down *TCF4* in A375/vem and SK-Mel-28/vem cells and found that the efficiency of

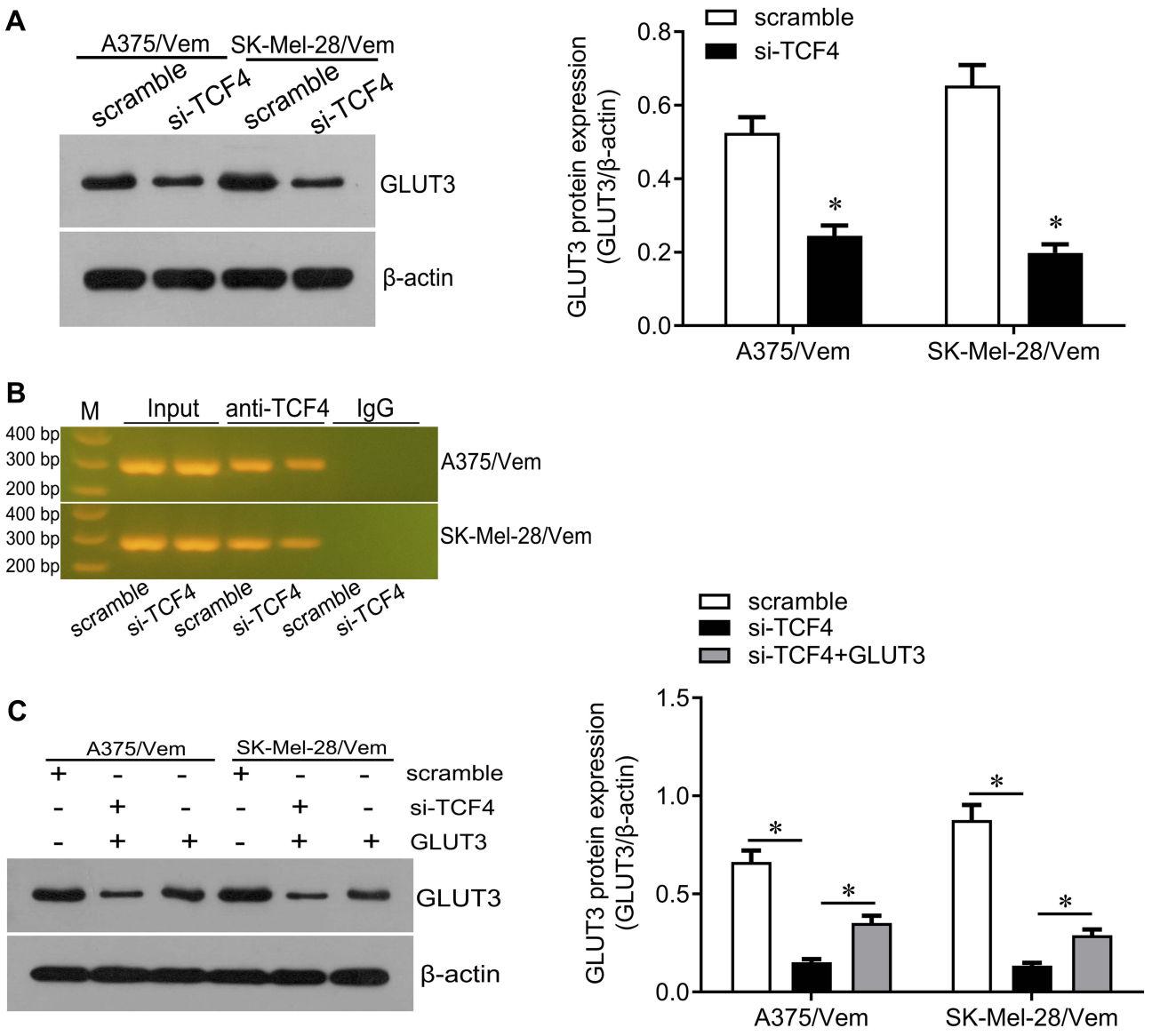

Figure 5 *TCF4* positively regulates *GLUT3*. (**A**) Western blot was performed to examine the expression of *GLUT3* in Vemurafenib-resistant A375 and SK-Mel-28 cells after siRNA transfection (left), and quantification of the bands (right). (**B**) Chip assay was performed to test the interaction between *TCF4* and *GLUT3*. (**C**) Western blot was performed to examine the expression of *GLUT3* in Vemurafenib-resistant A375 and SK-Mel-28 cells after *TFC4* siRNA and *GLUT3* expressed plasmids transfection (left), and quantification of the bands (right). Each experiment was performed three times. Vem, vemurafenib. *p<0.05.

siRNA-2 was higher than siRNA-1 (Figure 3A). The siRNA-2 was used for the next experiments. We found that *TCF4* knockdown significantly inhibited cell survival compared with scramble control in A375/vem and SK-Mel-28/vem cells under vemurafenib treatment (Figure 3B and C). In addition, *TCF4* knockdown suppressed the colony ability and promoted cell apoptosis in A375/vem and SK-Mel-28/vem cells without vemurafenib treatment (Figure 4A and B). Importantly, *TCF4* knockdown had great synergistic effect with vemurafenib in suppression of cell colony and promotion of cell apoptosis (Figure 4A and B).

GLUT3 Is a Downstream Molecule of *TCF4* in Melanoma Cells

We next investigated how *TCF4* sensitizes melanoma cells to vemurafenib. *GLUT3* plays a key role in glycolysis that promotes drug resistance in cancer cells. *TCF4* knockdown reduced the expression of *GLUT3* in A375/vem and SK-Mel-28/vem cells (Figure 5A). ChIP results showed that *TCF4* interacted with *GLUT3* (Figure 5B). We then rescued the expression of *GLUT3* in A375/vem and SK-Mel-28/vem cells that transfected with *TCF4* siRNA (Figure 5C). Rescue of *GLUT3* significantly

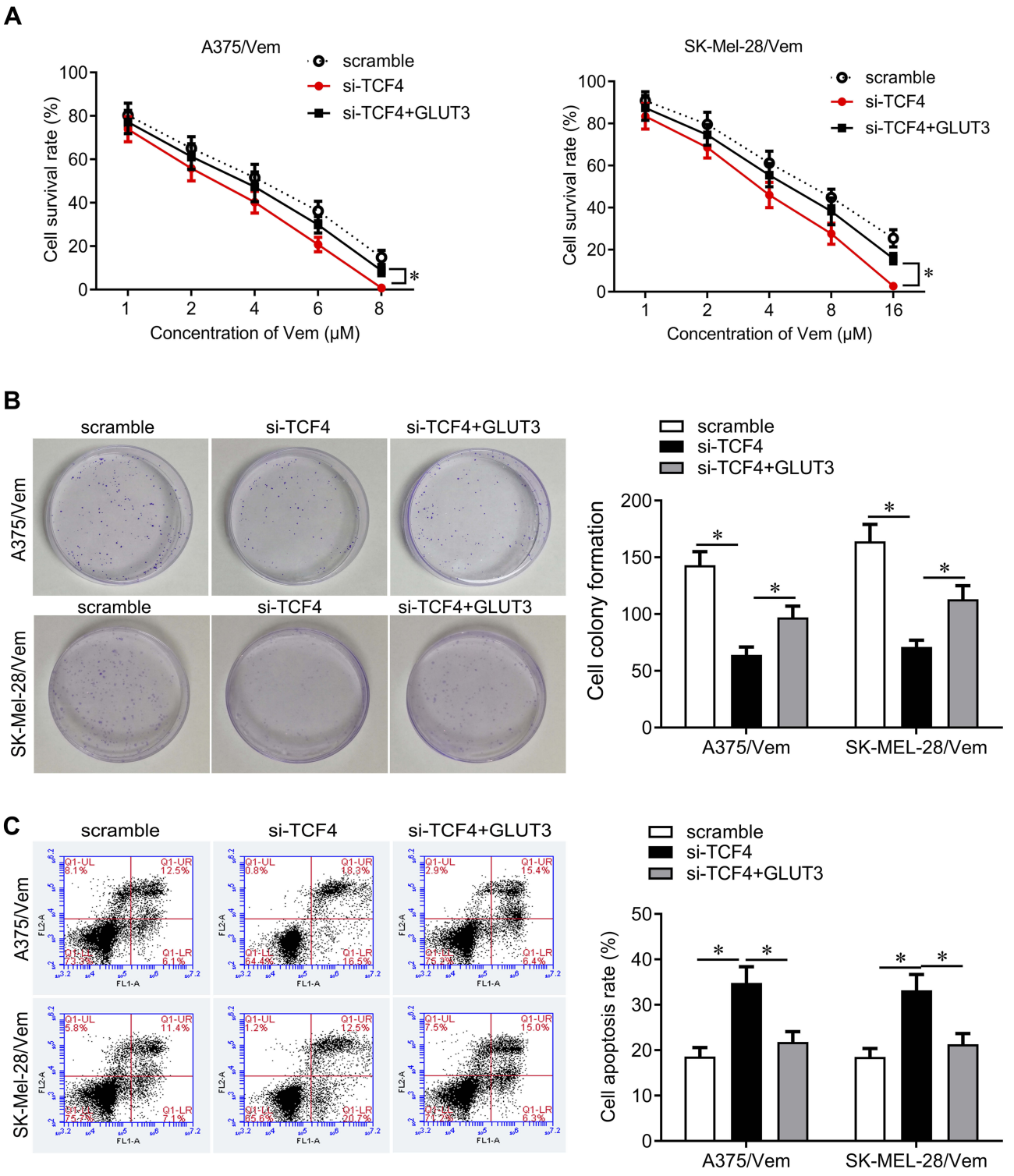

Figure 6 *GLUT3* reverses *TCF4* knockdown-mediated effects on cell proliferation and cell apoptosis. (**A**) CCK-8 assay was performed to measure the cell viability in A375/Vem and SK-Mel-28/Vem cells after *TFC4* siRNA and *GLUT3* expressed plasmids transfection. (**B**) Colony formation was performed to measure the ability of cell proliferation in A375/Vem and SK-Mel-28/Vem cells after *TFC4* siRNA and *GLUT3* expressed plasmids transfection. (**C**) Cell apoptosis was analyzed to measure the apoptotic rate in A375/Vem and SK-Mel-28/Vem cells after *TFC4* siRNA and *GLUT3* expressed plasmids transfection. Each experiment was performed three times. Vem, vemurafenib. *p<0.05.

attenuated the effects of *TCF4* knockdown on inhibition of cell survival and cell colony and on promotion of cell apoptosis (Figure 6A–C). In addition, *TCF4* knockdown inhibited the glycolysis process, evaluated by decrease in glucose uptake, ATP level and lactate production in A375/vem and SK-Mel-28/vem cells (Figure 7).

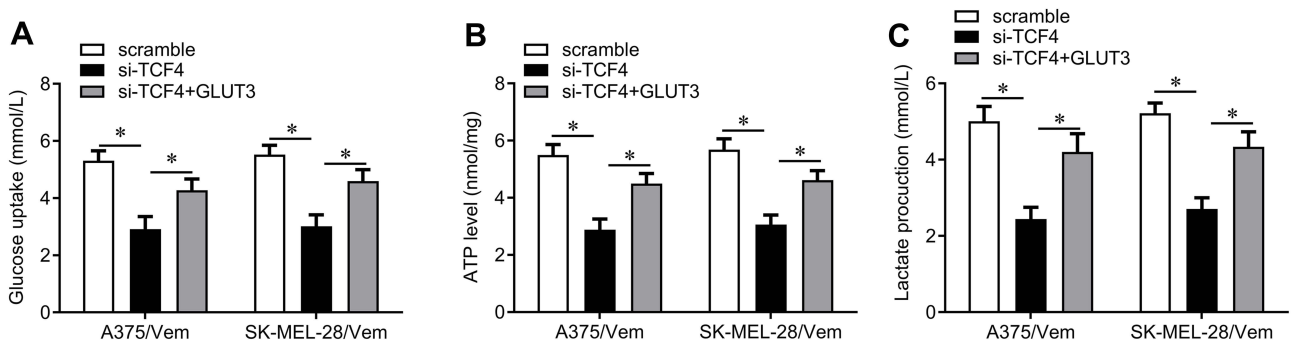

Figure 7 The effects of *TCF4*/*GLUT3* on glucose uptake, APT level and lactate production in Vemurafenib-resistant A375 and SK-Mel-28 cells. (**A**) Glucose Uptake Assay Kit was used to measure the glucose uptake in A375/Vem and SK-Mel-28/Vem cells after *TFC4* siRNA and *GLUT3* expressed plasmids transfection. (**B**) ATP Assay Kit was used to measure the ATP level in A375/Vem and SK-Mel-28/Vem cells after TFC4 siRNA and GLUT3 expressed plasmids transfection. (**C**) D-Lactate Assay Kit was used to measure the lactate production in A375/Vem and SK-Mel-28/Vem cells after *TFC4* siRNA and GLUT3 expressed plasmids transfection. Each experiment was performed three times. Vem, vemurafenib. *p<0.05.

However, rescue of *GLUT3* significantly abolished the effects of *TCF4* knockdown on glycolysis process (Figure 7).

Discussion

Melanoma is highly malignant with an annual growth morbidity of 3% to 5%. About 25% of melanoma patients in Asian countries have BRAF mutations. More than 90% of the BRAF mutations to valine at position 600 are replaced by glutamate (V600E), which cause the mitogen-activated protein kinase (MAPK) signaling pathway to lose control, resulting in uncontrolled cell growth.[14] Preclinical studies have shown that inhibiting BRAF by the small molecule RNAs can restore the cell growth cycle, thereby reversing the malignant phenotypes of melanoma cells.[15] Vemurafenib is a specific inhibitor of the BRAF gene V600E mutation, which can selectively block RAF/MEK/ERK signaling and suppresses tumor proliferation.[16] It has extensive safety and high selectivity. However, long-term single-dose therapy makes patients quickly resistant to Vemurafenib.[17] In this study, we found that *TCF4* expression was increased in Vemurafenib-resistant melanoma cells, and knocking down TCF4 could promote the sensitivity of melanoma cells to Vemurafenib. Studies have shown that activated *TCF4* significantly promotes tumor cell proliferation and metastasis in a variety of tumors. For example, *TCF4* gene knockout leads to early apoptosis of stem cells and affects the differentiation and proliferation of large intestinal crypt stem cells through the MAPK signaling pathway.[18] The expression level of *TCF4* in liver cancer tissues was significantly higher than that in adjacent tissues, and it was higher in metastatic liver cancer tissues. *TCF4* showed enhanced transcription activity, promoted the ability of cell clone formation and resistance to chemotherapy in liver cancer cells.[19] In addition, *TCF4* expression is increased in lung cancer, renal carcinoma, and breast cancer, which is related to the invasion and metastasis of tumor cells.[20,21]

In melanoma, *TCF4* is preferentially expressed by dedifferentiated/invasive phenotype cells.[22] *TCF4* is associated with epithelial–mesenchymal transition and invasion, induces the transcription of miR-125b to promotes melanoma cell invasion,[23] indicating that *TCF4* is associated with a more malignant phenotype. Previous study demonstrated that glucose transporter (GLUT) 3 was a direct *TCF4*/*β-catenin* target gene in hepatoblastoma, which is a *Wnt*/*β-catenin*-driven malignancy.[24] This study found that *TCF4* could interact with *GLUT3* and silencing *TCF4* could inhibit *GLUT3* expression. In addition, overexpression of *GLUT3* reversed the growth and glycolysis of tumor cells that were inhibited by *TCF4 k*nockdown. *GLUT* is a major carrier that mediates glucose transport inside and outside the cell, mainly distributed on the cell membrane.[25] There are 9 types of *GLUT, GLUT1-GLUT9*. *GLUT3* is responsible for the uptake of glucose for the nervous system.[26] More and more studies have found that *GLUT3* is overexpressed in various solid tumors, including ovarian cancer, gastric cancer, and non-small cell lung cancer.[27] This is because the rapid proliferation of tumor cells makes them in an anoxic environment. Under anaerobic conditions, ATP produced by glycolysis is more than 10 times less than aerobic metabolism. Therefore, tumor cells need to express a large number of *GLUTs* to meet the excess glucose uptake.[28] Melanoma cells have an obvious aerobic glycolytic phenotype, ie, Warburg effect.[29] *GLUT3* is highly expressed in melanoma and may become a potential

biomarker.[30] In addition, previous experiments have shown that some *GLUTs* transport inhibitors have shown good effects in inhibiting tumor growth in mice, making *GLUTs* a potential target for tumor therapy.[31]

Summary, this recent study demonstrates that *TCF4* downregulation sensitizes melanoma cells to Vemurafenib through inhibiting *GLUT3*-mediated glycolysis. These findings support *TCF4* as an oncogene and provide new mechanism by which *TCF4* confers chemotherapy resistance in melanoma.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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