Zwint facilitates melanoma progression by promoting c-Myc expression

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Abstract. ZW10 interactor (Zwint) is upregulated in various types of tumors and exerts a carcinogenic effect. However, little is known about the expression profile, function and molecular mechanisms of action of Zwint in melanoma. Therefore, the aim of the present study was to investigate the expression levels of Zwint in melanoma cell lines and tissues. It was revealed that Zwint was highly expressed in melanoma samples. Functional experiments indicated that Zwint knockdown suppressed the proliferation and migration of A375 melanoma cells. Further mechanistic studies demonstrated that Zwint knockdown decreased the protein expression levels of c-Myc, MMP-2, Slug, mTOR, phosphorylated (p)-mTOR, p-p38 and fibronectin, while it increased the protein expression levels of E-cadherin and MMP-9. Among these genes, c-Myc, MMP-2 and Slug were overexpressed to investigate their effects on cell proliferation following Zwint knockdown. The results demonstrated that overexpression of c-Myc, but not MMP-2 or Slug, rescued the effects of Zwint knockdown on melanoma cell proliferation and migration. Taken together, the results of the present study suggested that Zwint may act as an oncogene in melanoma by regulating c-Myc expression.

Introduction

Melanoma, which is derived from melanocytes, is the most aggressive type of skin cancer (1). According to epidemiological studies, the incidence and mortality rates of patients with melanoma are continuing to increase in the United States (2). Despite improvements in the diagnosis and treatment of melanoma, the prognosis of patients with advanced melanoma remains unfavorable, with a 3-year survival rate of only 15% (3,4). Therefore, it is critical to define the molecular mechanisms of action underlying melanoma growth and metastasis, with the aim to develop novel therapeutic strategies.

ZW10 interactor (Zwint) is a kinetochore protein found in higher eukaryotes, which plays an important role in the mitotic checkpoint (5,6). The interaction between Zwint and ZW10 mediates stable ZW10 kinetochore residency at prometaphase kinetochores, which is indispensable for mitotic checkpoint arrest (7,8). Given that the mitotic checkpoint is a primary mechanism ensuring high fidelity of segregation during mitosis, abnormalities often lead to the development of tumors (9). Therefore, it may be hypothesized that an abnormal expression or structure of Zwint may be associated with carcinogenesis. Previous studies have reported that Zwint is overexpressed in various types of tumors and is associated with a poor prognosis (10-12). In addition, it has been demonstrated that Zwint may act as an oncogene (11,13). However, whether Zwint is associated with melanoma progression and its specific molecular mechanism of action remain unclear.

The aim of the present study was to investigate whether Zwint regulates melanoma growth and metastasis and whether its effects are mediated by promoting c-Myc expression, in order to determine whether Zwint may serve as a novel target for the treatment of melanoma.

Materials and methods

Data Download. Microarray data set (GSE46517) (14) was downloaded from Gene Expression Omnibus (ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46517). The expressing level of Zwint in 31 primary melanoma samples and 9 nevi tissues were from GSE46517 (GPL96 Affymetrix Human Genome U133A Array).

Cell culture. The human melanoma cell lines A375, A2058 (amelanotic melanoma) and 451Lu (metastatic melanoma) were purchased from GeneChem, Inc. A375 and A2058 were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.). 451Lu cell lines were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.). The immortalized normal human epidermal melanocyte cell line, PIG1 (a gift from Dr Caroline Le Poole, Loyola University Chicago, USA), was cultured in Medium 254 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with

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Human Melanocyte Growth Supplement (Invitrogen; Thermo Fisher Scientific, Inc.) and 5% FBS (Invitrogen; Thermo Fisher Scientific, Inc.).

Plasmids, short hairpin (sh)RNA and transfection. The lenti-plasmid vector system CV224 (Ubi-MCS-SV40-Cherry-IRES-puromycin) and lenti-shRNA vector system GV115 (hU6-MCS-CMV-EGFP) were constructed, packed and purified by GeneChem, Inc. A second generation system was used for lentivirus construction. Zwint targeting shRNA-1 (5'-CCGGCAGAGAATCTTCCAGAT GATACTCGAGTATCATCTGGA AGATTCTCTGTTT TTG-3') or shRNA-2 (5'-CCGGCTACAACCTGCTGGAGA TGTACTCGAGTACATCTCCAGCAGGTTGTAGTTTTT G-3') and control shRNA (5'-CCGGCCTTCTCCGAACGT GTCACGTCTCGAGTAAATGCTGGTACTCAAATGGTT TTT-3') were designed with a hairpin and inserted into the GV115 vector. c-Myc cDNA, MMP-2 cDNA and Slug cDNA were cloned into CV224 vector and empty vector was used as negative control. Then, 293T cells (Cell Bank of the Chinese Academy of Sciences) were seeded into a 10 cm dish (5×10^6 cells) and transfected using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The vectors were transfected in the following proportions: GV/CV vector plasmid: pHelper1.0 vector plasmid: Phelper 2.0 vector plasmid; 20 μg: 15 μg: 10 μg for 48 h at 37°C in an incubator before the 293T lentiviral supernatant was harvested by centrifugation at 4,000 x g for 10 min at room temperature. Phelper 1.0 vector contains the *gag* gene of HIV virus, encoding the main structural proteins of the virus; the *pol* gene, encoding a virus-specific enzyme; the *rev* gene encodes regulatory factors that regulate the expression of *gag* and *pol* genes. Phelper 2.0 vector contains the herpes simplex virus-derived VSV-G gene, which provides the envelope protein needed for virus packaging. The lentiviral supernatant was concentrated and purified for further concentration detection, and 10 MOI was used for the next transfection. A375 cells were seeded into a six-well plate at a density of 1×10^5 cells/well and were infected with the lenti-shRNA vectors alone or co-infected with lenti-plasmid-Cherry vectors for 24 h. After that, culture medium containing the virus was replaced with fresh culture medium. Cells were collected 72 h after infection for further analysis. The expression levels of the reporter gene Cherry or GFP were observed using fluorescence microscopy (Olympus Corporation; magnification, x100). If the fluorescence rate was >70% and the cells were considered healthy, subsequent experiments were performed.

Reverse transcription-quantitative (RT-q)PCR. Total RNA extracted from the melanoma cells was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR experiments were performed using the SYBR-Green Premix Ex Taq kit (Takara Biotechnology, Co., Ltd.) according to the manufacturer's protocol. BIO-RAD MJ Mini Opticon Real-Time PCR System was used to determine the relative mRNA levels. The following thermocycling conditions were used: Initial denaturation at 95°C for 30 sec, 40 cycles at 95°C for 5 sec, 60°C for 30 sec and 72°C for 20 sec. Threshold cycle values were used to calculate the fold-change in the transcript levels by using the $2^{-\Delta\Delta Cq}$ method (15). The relative mRNA expression levels were normalized to the GAPDH gene. The following primer sequences were used: Zwint forward, 5'-CACGTAGAGGCC ATCAAAATTGG-3' and reverse, 5'-CGGAGTTGTGTCCGT TTCCT-3; GAPDH forward, 5'-TGACTTCAACAGCGACAC CCA-3' and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'; MMP-2 forward, 5'-GATACCCCTTTGACGGTAAGGA-3' and reverse, 5'-CCTTCTCCCAAGGTCCATAGC-3'; Slug forward, 5'-GCCAAACTACAGCGAACTGG-3' and reverse, 5'-ATTGGGTAGCTGGGCGTGGA-3'; c-Myc forward, 5'-TGGTGCTCCATGAGGAGACA-3'; and reverse, 5'-TGT GAGGAGGTTTGCTGTGG-3'.

Western blot analysis. Western blot analysis was performed as previously described (16). Melanoma cells were washed with phosphate buffered saline (PBS) and lysed using RIPA lysis buffer (Xi'an Runde Biotechnology Co., Ltd.) and phenylmethanesulfonyl fluoride (protease inhibitor mix; Sigma-Aldrich; Merck KGaA). Cell lysates were cleared by centrifugation for 15 min at 13,000 x g at 4°C. Total protein was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and 20 μg protein/lane was separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Following blocking in a solution of 5% non-fat dry milk diluted in tris-buffered saline (TBS; pH 7.4) containing 0.05% Tween-20 at room temperature for 1 h, the membranes were washed and incubated with primary antibodies overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bound antibodies were detected using a chemiluminescence detection kit (KPL, Inc.). Primary antibodies against Zwint (1:1,000; cat. no. ab71982), c-Myc (1:1,000; cat. no. ab32072), Twist (1:1,000; cat. no. ab50887), p-mTOR (1:2,000; cat. no. ab109268), N-Cadherin (1:1,000; cat. no. ab18203), Fibronectin (1:500; cat. no. ab32419), and GAPDH (1:2,000; cat. no. ab37168) were purchased from Abcam. Antibodies against Slug (1:1,000; cat. no. 9585), MMP-2 (1:500; cat. no. 13132), p-AKT (1:1,000; cat. no. 4060), Snail (1:2,000; cat. no. 3879), AKT (1:2,000; cat. no. 4691), β-catenin (1:2,000; cat. no. 8480), NF-κB p65 (1:2,000; cat. no. 8242), Vimentin (1:1,000; cat. no.3932), p-NF-κB p65 (1:1,000; cat. no. 3033), p-p38 (1:1,000; cat. no. 4631), MMP-9 (1:500; cat. no. 13667), p38 (1:2,000; cat. no. 8690), p-β-catenin (1:1,000; cat. no. 2009), mTOR (1:500; cat. no. 2972), ERK (1:2,000; cat. no. 9102), E-Cadherin (1:2,000; cat. no. 9107) and p-ERK (1:2,000; cat. no. 4376) were purchased from Cell Signaling Technology, Inc. Secondary antibodies including anti-rabbit IgG (1:10,000; cat. no. AS1107) or anti-mouse IgG (1:10,000; cat. no. AS1106) were purchased from ASPEN Biotechnology CO., LTD.

Cell proliferation assay. Following transduction, A375 cells from each group were trypsinized and counted. Cells were seeded into 96-well plates at a density of 2×10^3 cells/well in quintuplicate and incubated at 37°C for 1, 2, 3, 4 and 5 days. Cell numbers were counted over the 5 continuous days.
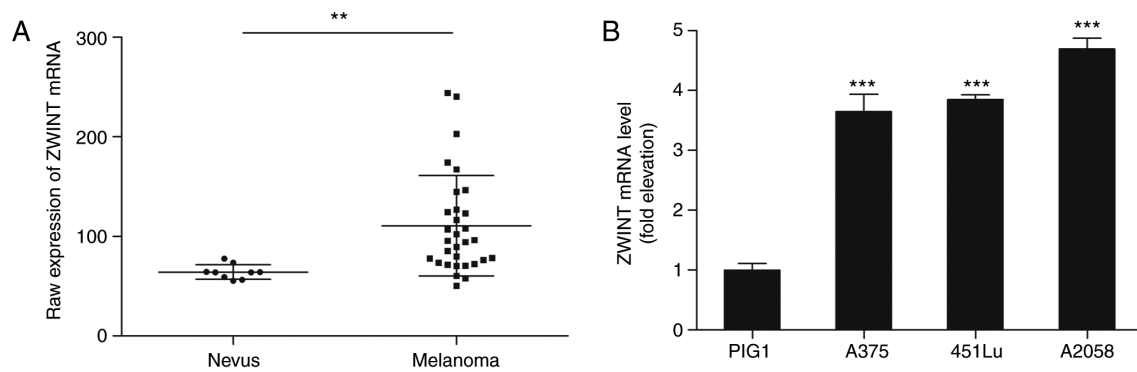
Figure 1. Zwint mRNA is highly expressed in melanoma tissues and cells. (A) Zwint mRNA expression was upregulated in melanoma tissues (n=31) compared with nevus tissues (n=9), according to the GSE46517 dataset. (B) Reverse transcription-quantitative PCR analysis in different melanoma cell lines indicated that Zwint expression levels were increased in melanoma cells compared with PIG1 normal human epidermal melanocytes. **P<0.01 vs. nevus; ***P<0.001 vs. PIG1. ZWINT, ZW10-interactor.

Fluorescence microscopy was used to assess the cell counts using Celigo Imaging Cytometer (Nexcelom Bioscience LLC; magnification, x100). Cell proliferation was also assessed using MTT assay (Beijing Dingguo Changsheng Biotechnology Co., Ltd.). Following transduction, cells were incubated with 20 μl MTT at 37°C for 4 h. Following MTT incubation, the purple formazan crystals were dissolved using DMSO and cell proliferation was subsequently analyzed at a wavelength of 490 nm using a microplate reader (infinite M2009PR; Tecan Group, Ltd.).

Transwell assay. Following transduction, A375 cells were trypsinized and resuspended in serum-free DMEM at a density of 1x10^5 cells/ml. Cell suspensions (100 μl) were plated in the upper chambers (pore size, 8 μm; Corning, Inc.), while 600 μl DMEM supplemented with 30% FBS was plated in the lower chambers. Following incubation at 37°C for 24 h, the migratory cells were fixed with 4% paraformaldehyde for 30 min at room temperature and subsequently stained with 4 g/l crystal violet for 10 min at room temperature. Stained cells were counted in nine randomly selected fields using a light microscope (Olympus Corporation; magnification, x100).

Statistical analysis. Statistical analysis was performed using GraphPad Prism v5.00 software (GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± SD. Unpaired student's t-test was used to compare differences between two groups. One-way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Zwint expression in melanoma. To determine the role of Zwint in melanoma, the GSE46517 dataset was downloaded from the Gene Expression Omnibus database. The analysis demonstrated that Zwint expression was significantly upregulated in primary melanoma lesions compared with nevus samples (P=0.00178; Fig. 1A). Subsequently, the mRNA level of Zwint was examined in various melanoma cell lines (A375, 451Lu and A2058) as well as in the immortalized normal human epidermal melanocyte cell line PIG1. The mRNA level of Zwint in all melanoma cell lines was increased compared with that in the PIG1 cells (Fig. 1B).

Zwint knockdown suppresses the proliferation and migration of melanoma cells. To investigate the effect of Zwint knockdown on the malignant growth potential, A375 cells were transfected with two different shRNAs targeting Zwint. Western blot and RT-qPCR analyses demonstrated that Zwint expression significantly decreased following transfection with both shRNAs compared with the control shRNA (Fig. 2A and B). The proliferation of melanoma cells was assessed via cell counting and MTT assay. The results demonstrated that Zwint knockdown notably decreased cell proliferation (Fig. 2C and D). The ability of Zwint to modify the motility of melanoma cells was also assessed. The results of the Transwell migration assay demonstrated that A375 cells transfected with either Zwint shRNA-1 or shRNA-2 migrated at a slower rate compared with the control cells (Fig. 2E). Notably, the inhibitory effect on cell proliferation and migration was more pronounced in A375 cells transfected with Zwint shRNA-1 compared with Zwint shRNA-2. Thus, Zwint shRNA-1 was selected for subsequent experiments.

Rescue of c-Myc in Zwint knocked-down cells restores cell proliferation and migration. Given the notable effects of Zwint knockdown on melanoma cells, the signaling pathways involved in tumor growth and migration were assessed via western blot analysis. The results demonstrated that the expression levels of c-Myc, MMP-2, Slug, mTOR, phosphorylated (p)-mTOR, p-p38 and fibronectin were decreased following Zwint knockdown, while the expression levels of E-cadherin and MMP-9 were increased. However, the expression levels of ERK, p-ERK, N-cadherin, Snail, AKT, p-AKT, β-catenin, p-β-catenin, p38, NF-κB p65, p- NF-κB p65, Twist and Vimentin did not change following Zwint knockdown. (Fig. 3A-H). To identify which gene may serve a crucial role in mediating the function of Zwint, MMP-2, Slug and c-Myc were overexpressed to investigate their effects on cell proliferation and migration following Zwint knockdown. The overexpression efficiency of MMP-2, Slug and c-Myc vectors was verified by western blotting (Fig. S1). Furthermore,
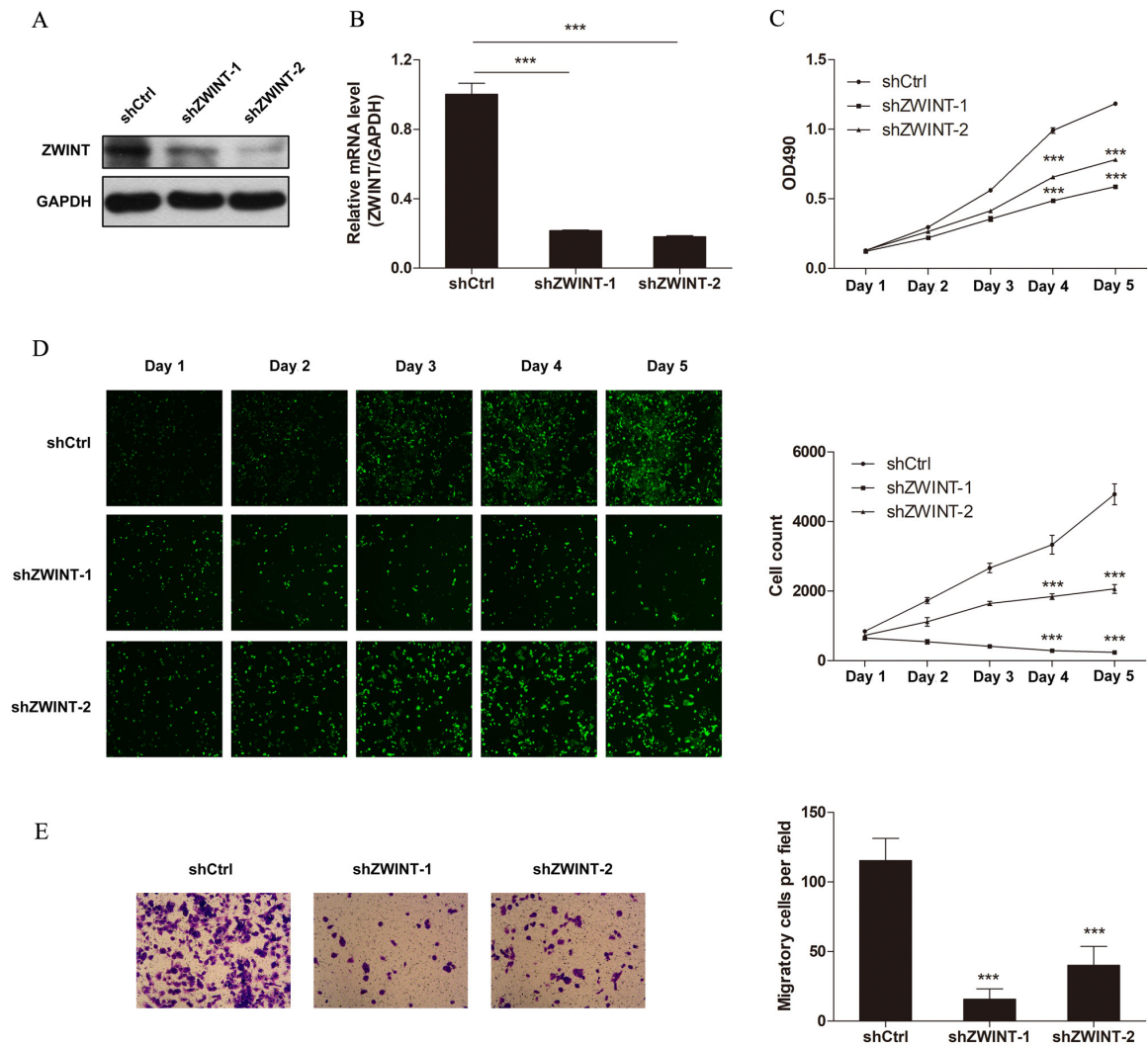
Figure 2. Zwint knockdown suppresses the proliferative and migratory ability of A375 melanoma cells. (A) Western blotting and (B) reverse transcription-quantitative PCR analyses were performed to detect the knockdown efficiency of two different shRNAs against Zwint. (C) MTT assay indicated that knockdown of Zwint inhibited A375 cell proliferation. (D) Control or Zwint knocked-down A375 cells expressing green fluorescent protein were visualized to perform cell counts using a Celigo Imaging Cytometer (magnification, x100). (E) Transwell assay demonstrated that knockdown of Zwint significantly inhibited the migration of A375 cells (magnification, x100). ***P<0.001 vs. shCtrl. OD, optical density; sh; short hairpin; Ctrl, control; ZWINT, ZW10-interactor.
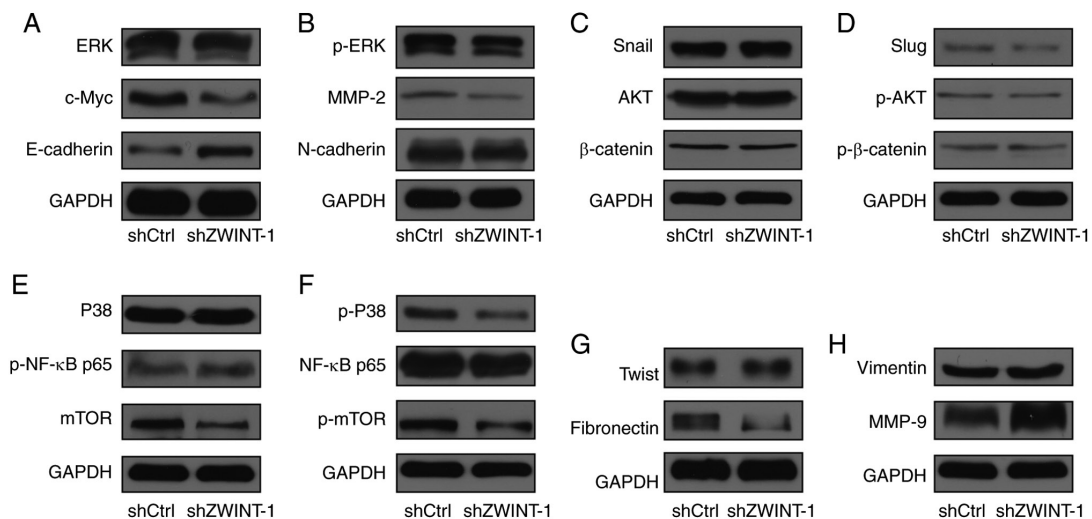

Figure 3. Screening for the downstream effectors regulated by Zwint. (A-H) Western blot analysis was performed to detect the protein expression changes of (A) ERK, c-Myc and E-cadherin; (B) p-ERK, MMP-2 and N-cadherin; (C) Snail, AKT and β-catenin; (D) Slug, p-AKT and p-β-catenin; (E) p38, p-NF-κB and mTOR; (F) p-p38, NF-κB and p-mTOR; (G) Twist and fibronectin; and (H) vimentin and MMP-9, following Zwint knockdown in A375 melanoma cells. p, phosphorylated; sh, short hairpin; Ctrl, control; ZWINT, ZW10-interactor.
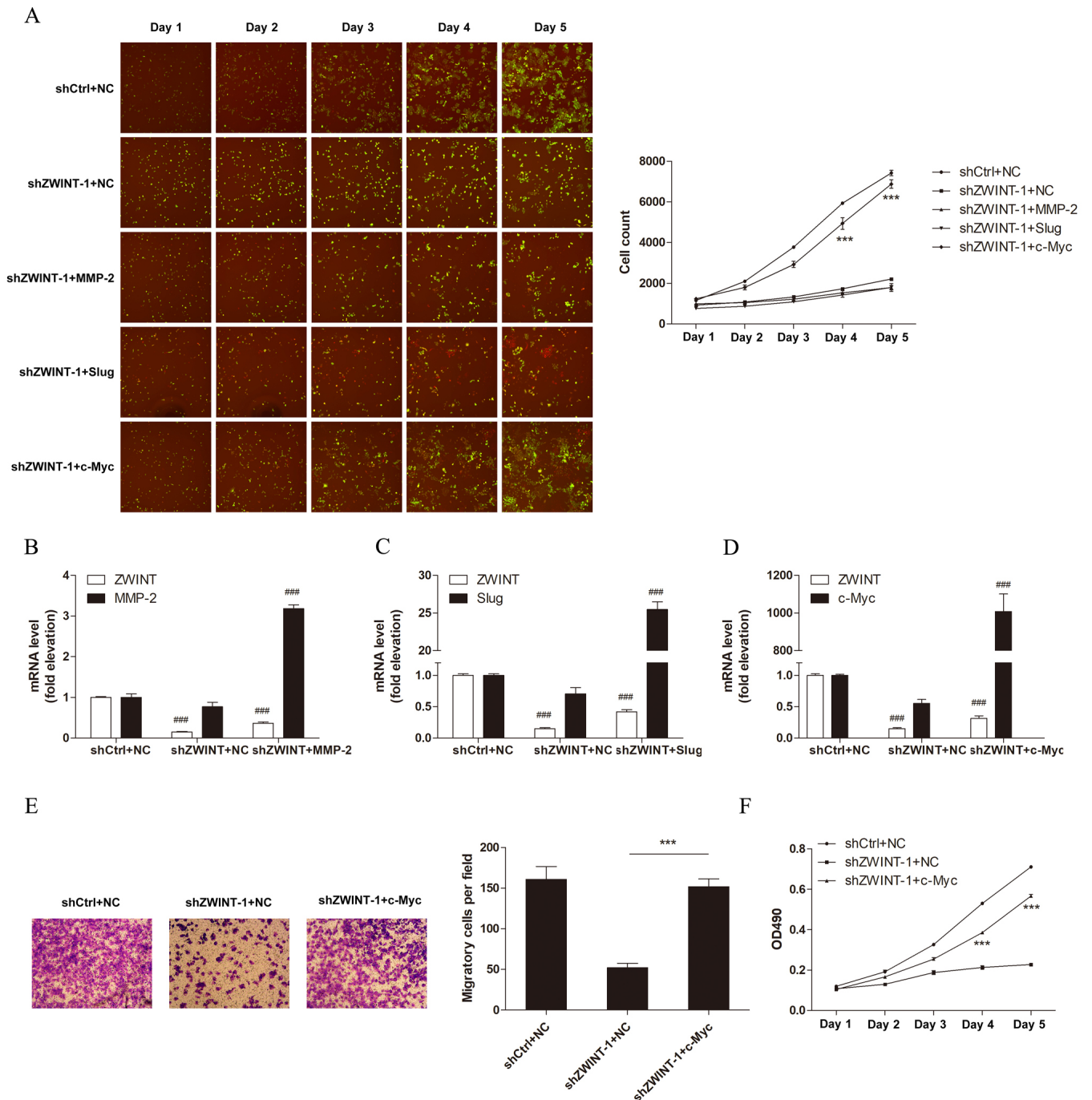
Figure 4. Rescue of c-Myc expression in Zwint depleted cells restores the proliferative and migratory abilities of A375 melanoma cells. (A) Celigo Imaging Cytometer was used to perform cell counts via fluorescence measurement in the indicated groups. Co-transfection of GFP-tagged shCtrl with Cherry-tagged NC, GFP-tagged shZWINT-1 with Cherry-tagged NC or Cherry-tagged MMP-2 or Cherry-tagged Slug or Cherry-tagged c-Myc in A375 cells (magnification, x100). (B-D) Reverse transcription-quantitative PCR analyses were performed to detect interference and overexpression efficiency following transfection with Zwint shRNAs and (B) MMP-2, (C) Slug or (D) c-Myc plasmids. (E) Transwell assay was performed to assess cell migration (magnification, x100). (F) MTT assay was performed to assess cell proliferation. ***P<0.001 vs. shZWINT-1 + NC. ###P<0.001 vs. shCtrl + NC. GFP, green fluorescent protein; NC, negative control; sh; short hairpin; Ctrl, control; ZWINT, ZW10-interactor.

Zwint knockdown or MMP-2, Slug and c-Myc overexpression efficiency was verified by RT-qPCR (Fig. 4B-D). The results of the cell counting tests demonstrated that c-Myc overexpression promoted cell proliferation in Zwint depleted cells compared with cells transfected with the empty vector (Fig. 4A), suggesting that c-Myc may be a downstream effector of Zwint. Furthermore, the results of the MTT and Transwell migration assays demonstrated that c-Myc overexpression in

Zwint depleted cells restored melanoma cell migration and proliferation (Fig. 4E and F).

Discussion

The present study aimed to investigate the expression and function of Zwint in melanoma and elucidate its molecular mechanism. The results demonstrated that Zwint was highly

expressed in melanoma tissues and cells. In addition, Zwint depletion suppressed melanoma cell proliferation and migration. Consistent with these results, previous studies have demonstrated that Zwint is highly expressed in different types of cancer and exerts an oncogenic effect. For example, Zhou *et al* (17) reported that Zwint is upregulated in breast cancer tissues compared with adjacent normal tissue and can promote breast cancer cell proliferation. Furthermore, Peng *et al* (13) demonstrated that Zwint is overexpressed in lung adenocarcinoma compared with adjacent normal tissue, and Zwint knockdown suppresses tumor malignant behaviors, such as proliferation, migration, invasion and colony formation. Upregulated Zwint expression has also been observed in human hepatocellular carcinoma, ovarian cancer and breast cancer compared with normal adjacent tissue and has been associated with a poor prognosis (11,12,17). Consistent with previous findings, the results of the present study suggested that Zwint acts as an oncogene and may be a potential therapeutic target in melanoma. However, the molecular mechanism underlying its carcinogenic effects remains to be fully elucidated.

To further investigate the molecular mechanism of Zwint in melanoma, western blot analysis was performed to screen downstream molecules associated with cell proliferation and migration, following Zwint knockdown. These downstream candidates included key molecules in proliferation-associated MAPK and PI3K-AKT signaling pathways (18,19), such as ERK, p-ERK, p38, p-p38, c-Myc, AKT, p-AKT, NF-κB p65, p-NF-κB p65, mTOR and p-mTOR. The candidates also included molecules associated with cell adhesion and extracellular matrix that promote tumor migration (20) such as Twist, Fibronectin, Snail, β-catenin, p-β-catenin, Slug, Vimentin, MMP-2, MMP-9, E-Cadherin and N-Cadherin. The results demonstrated that the expression levels of c-Myc, MMP-2, Slug, mTOR, p-mTOR, p-p38 and fibronectin were decreased following Zwint knockdown, while the expression levels of E-cadherin and MMP-9 were increased. Furthermore, subsequent experiments indicated that overexpression of c-Myc rescued the effects of Zwint depletion on melanoma cell proliferation and migration. Taken together, these results suggested that Zwint acts as an oncogene in melanoma by regulating c-Myc expression. However, whether other molecules regulated by Zwint also play a role in this process remains to be investigated. Zwint serves an important role in the spindle assembly checkpoint (7,8), which suggests that it may play a role in the pathogenesis of tumors. In addition to its role in the mitotic spindle checkpoint, increasing evidence has suggested that Zwint may participate in other signaling pathways. For instance, Zwint expression was indicated to be associated with the expression levels of PCNA, Cdc25C, cyclin B1 and CDK1 (11). Li *et al* (10) reported that high Zwint expression was closely associated with cell cycle-related molecules and pathways, such as Myc targets V1/2, DNA repair, mitotic spindle, G2M checkpoint and E2F targets. In addition, Peng *et al* (13) demonstrated that Zwint knockdown affected the expression of molecules associated with TNF signaling and other pathways. However, these potential molecular mechanisms of action of Zwint have not been confirmed by subsequent experiments. To the best of our knowledge, the present study was the first to demonstrate that Zwint promotes the malignant phenotype of melanoma by regulating c-Myc expression, which also supports previous findings with regards to the association between Zwint expression and the Myc pathway in bioinformatics analysis of gene expression profiles in breast cancer (10).

Dysregulation of the c-Myc oncogene has been observed in different types of tumor, such as liver and colon cancer, as well as Burkitt's lymphoma (21) and is known to contribute to the development of melanoma and other types of human cancer (22-24). The molecular mechanism regulating c-Myc expression is intricate. In addition to gene aberrances, such as genetic amplifications, insertions and translocations (25-27), a variety of factors can affect c-Myc expression. For example, Jiang *et al* (28) reported that TIP30 can repress estrogen receptor-α-mediated c-Myc transcription. Furthermore, Sachdeva *et al* (29) demonstrated that microRNA-145 can inhibit c-Myc expression at the post-transcriptional level. c-Myc expression is also regulated by acetylation and ubiquitination (30-32). However, the specific molecular mechanism underlying Zwint regulation of c-Myc expression remains unclear and requires further study. Given that c-Myc is considered 'undruggable' at the protein level (33), Zwint, as a regulator of c-Myc, may be of value as a novel target for the treatment of melanoma.

In conclusion, the results of the present study demonstrated that Zwint expression was upregulated in melanoma and promoted tumor progression. Furthermore, depletion of Zwint was indicated to impair melanoma cell proliferation and migration, potentially by downregulating c-Myc expression. Taken together, these results provided novel insights into the molecular mechanism of melanoma development and suggested that Zwint may constitute a promising target for melanoma therapy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KM and RG conceived the experiments. KM, JZ and LW performed most of the experiments. KM and RG confirm the authenticity of all the raw data. XM and WL helped analyze the data. All authors discussed the results and RG drafted the initial manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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