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FOXN3 Regulates Autophagic Activity to Suppress Drug Resistance in Melanoma Cells

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Background: The forkhead box (FOX) family member FOXN3 has been reported to inhibit transcriptional activity associated with regulating tumor development. However, the role of FOXN3 in the pathogenesis of melanoma is not well understood.

Objective: To investigate the biological functions of FOXN3 in drug resistance of melanoma.

Materials and Methods: The expression of FOXN3 in melanoma was investigated using Gene Expression profiling interactive analysis (GEPIA) and Linkedomics databases. Melanoma cell proliferation, invasion, and migration were assessed using the colony formation assay, the scratch wound healing test, the Transwell invasion assay, and the nude mice xenograft to determine the effects of FOXN3 over-expression and depletion. The functional role of the transcriptional regulator in melanoma cells was tested through chromatin immunoprecipitation, immunofluorescence.

Results: FOXN3 was downregulated in melanoma. Over-expression of FOXN3 inhibited the proliferation and motility of melanoma cells, whereas FOXN3 knockdown significantly enhanced the proliferation and motility of melanoma cells. Overexpression of FOXN3 reduced autophagic activity in melanoma cells. Enhanced autophagic activity in drug-resistant melanoma cell lines is related to drug-sensitive cells, and significant differences in FOXN3 localization were observed when comparing melanoma cells that were sensitive and resistant to Vemurafenib. Additionally, FOXN3 has been identified as binding to the promoter region of the cancer antigen Fibrous Sheath Interacting Protein 1 (FSIP1), thereby regulating the expression of this gene.

Conclusion: FOXN3 functions as an important regulator of the development and progression of Vemurafenib-resistant melanoma cells, partly owing to its binding to the *FISP1*. As such, FOXN3 may represent a relevant target for therapeutic interventions in patients suffering from drug-resistant melanoma.

Plain language summary: FOXN3, a member of the forkhead box (FOX) family, plays a role in inhibiting transcriptional activity linked to tumor development. However, its involvement in melanoma pathogenesis remains unclear. Our study explores FOXN3's functions in melanoma drug resistance, revealing reduced FOXN3 expression in melanoma tissue compared to normal skin. FOXN3 inhibits melanoma cell proliferation, invasion, and migration, leading to in vivo growth retardation. Overexpression of FOXN3 suppresses autophagy in drug-resistant melanoma cells, making it a key regulator in Vemurafenib-resistant melanoma progression. This suggests FOXN3 as a potential therapeutic target for drug-resistant melanoma patients.

Keywords: melanoma, FOXN3, autophagy, Vemurafenib, drug resistance

Introduction

Melanoma is one of the most prevalent and lethal forms of cancer due to its high metastatic progression rates.[1,2] With the advent of immunotherapies and combination treatment regimens, significant advances have been made in treating melanoma over the past few decades.[1] However, further research is required to improve patient prognosis, and many

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individuals fail to respond to immunotherapeutic intervention.[3] These treatment failures may be associated with target antigens not being expressed by all melanoma cells within a given patient, enabling some level of therapeutic escape.[4]

Mutations in the BRAF gene are a common finding in patients with melanoma, with the V600E mutation contributing to constitutive MAPK signaling activity in malignant melanoma cells being the most prevalent.[5] Specifically, constitutively activated BRAF can phosphorylate MEK, activating it and driving the downstream phosphorylation of ERK.[6] This pathway can ultimately alter the expression and activity of a diverse array of transcriptional regulators that ultimately shape tumor cells' proliferation, survival, and differentiation. The small molecule BRAF inhibitor Vemurafenib has been reported to exhibit good initial clinical responses in treated patients, but the emergence of acquired Vemurafenib resistance ultimately leads to poor long-term efficacy.[7] In general, the pathways thought to contribute to melanoma, and other kinase-driver tumor cell resistance to Vemurafenib include the activation of alternative signaling pathways that bypass the MAPK pathway, the reactivation of such MAPK signaling, or other poorly defined mechanisms.[8] The connection between autophagy and melanoma resistance to targeted therapy is increasingly supported by data.[9,10] Autophagy plays a pivotal role in cell survival during stress, involving the formation of autophagosomes and autolysosomes, which recycle macromolecules for energy.[11] Recent evidence also suggests a link between secretory autophagy, exosomes, and modulation of resistance and antitumor responses.[12,13]

Fibrous sheath interacting protein 1 (FSIP1) is located on chromosome 15q14, which is cancer/testis antigen and bound to A-kinase anchoring protein.[14,15] Studies assessing FSIP1 have highlighted the potential importance of FSIP1 in several cancer types. It has been shown to enhance the activity of key signaling pathways involved in melanoma progression. Furthermore, Chen et al reports that loss-of-function variants in FSIP1 identified by targeted sequencing are associated with one particular subtype of mucosal melanoma.[16] At present, a comprehensive investigation into the intricate regulatory mechanisms underlying the action of FISP1 in melanoma remains elusive.

The forkhead box (FOX) transcription factor family member FOXN3 is a key regulator of cellular proliferation, migration, and invasivity.[17,18] FOXN3 has recently been recognized as a key mediator of many cancers, including melanoma,[18] papillary thyroid,[19] adult acute myeloid leukemia,[20] and breast cancer.[21] However, the specific roles played by FOXN3 and FSIP1 in melanoma remain to be fully defined. Accordingly, this study was designed to assess the biological functions of FOXN3 and its potential impact on the transcriptional activity of FSIP1 in melanoma to elucidate any potential relationship between this transcription factor and the emergence of tumor cell resistance to Vemurafenib.

Materials and Methods

Analyses of the GEPIA Database

The GEPIA database (http://gepia.cancer-pku.cn/detail.php) is an online resource that allows for the analyses of RNA-seq data pertaining to 9736 tumor and 8587 control samples included in the Genotype-Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) databases and Xiantao tools (https://www.xiantaozi.com)[22]. This GEPIA database was used to assess differentially expressed genes (DEGs) associated with melanoma ($|\log2FC| > 1$, $P < 0.05$) for this study, with gene expression box plots generated automatically following the entry of gene symbols into the appropriate column. Using this database, differences in gene expression as a function of tumor stage were also evaluated.

Analyses of the LinkedOmics Database

The LinkedOmics database is a public platform, providing comprehensive multi-omics data from 11,158 patients for 32 types of cancer.[23] The LinkedOmics database (http://www.linkedomics.orglogin.php), which contains RNA-seq data from 103 melanoma patients in the TCGA database, was employed to identify co-expressed genes with FOXN3. In the current study, FOXN3-related differentially expressed genes (DEGs) were screened in melanoma samples using the "LinkFinder" module. The correlation results were tested by the Pearson correlation analysis and visualized via volcano and heat maps. The FOXN3-related DEGs were annotated using Gene Ontology biological process (GO_BP) in the "Function" module. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was analyzed by the gene set enrichment analysis (GSEA) using the "LinkInterpreter" module.

Gene Set Enrichment Analysis (GSEA)

The GSEA approach enables the large-scale detection of changes in gene expression and associated pathways based on TCGA gene expression datasets.[24] Student's *t*-test scores are computed for consistent pathways and mean values for DEGs are computed in individual analyses. Hallmark pathways exhibiting significant enrichment were detected through 1000x permutation tests. Results were adjusted for multiple testing through false discovery rate (FDR) and Benjamini and Hochberg (BH) correction to generate an adjusted *P*-value, with an adjusted $P < 0.01$ and an FDR < 0.25 established as the criteria for identifying significantly involved genes.

Cell Lines and Cell Transfection

Normal human HaCaT cells obtained from Shanghai Cell Bank of the Chinese Academy of Science, along with four different human melanoma cell lines (A375 from American Type Culture Collection, HMY-1, SK-MEL-1, and A875 from Kunming Cell Resource Center of Chinese Academy of Sciences), were cultured in DMEM (GE Healthcare, Little Chalfont, UK) supplemented with 4 mm L-glutamine, 4.5 g/L glucose and 10% FBS (Gibco Invitrogen, CA, USA). Authentication of these cell lines was performed using STR analyses to ensure their reliability and reproducibility.

A375 and A875 cell lines were transfected with Lipofectamine 3000 (Invitrogen, CA, USA) based on provided directions. Overexpression studies were conducted using GV141 plasmids carrying FOXN3 expression vectors in A375 cells, while downexpression studies utilized FOXN3-specific shRNA-expressing plasmids (shR1 and shR2) in A875 cells. Subsequent to transfection, A375 cells expressed exogenous FOXN3 protein, whereas A875 cells exhibited suppressed endogenous FOXN3 expression due to shRNA transfection. At 48 h after transfection, Western immunoblotting was used to assess the efficiency of transfection.

Cell Viability, Invasion and Colony Formation Assay

Functional assays were then employed to assess the impact of altered FOXN3 expression levels on cellular behavior in both contexts, providing insights valuable for understanding FOXN3's role in melanoma progression and potential therapeutic interventions. Cell viability was detected by CCK-8. CCK-8 reagent was added to the culture medium at 24, 48, 72, and 96 hours and the OD value was measured at 450 nm. Transwell inserts (Millipore, USA) were used to measure cell invasion after being precoated with Matrigel (BD Biosciences) following the manufacturer's recommendations.

The transfection of FOXN3 expression vectors into A375 cells or the transfection of shR1 into A875 cells was followed by colony formation assays to evaluate their capacity to form colonies. A375 and A875 cells were inoculated at a density of 2×10^5 cells/well. During colony growth, the culture medium was replaced every 3 days, and the cells were further incubated for 6 days to allow for significant colony formation. The formed colonies were then fixed with 4% tissue fixative for 15 minutes, followed by staining with 1% crystal violet solution for an additional 15 minutes. Finally, the colonies were washed with PBS to remove excess dye. This procedure was repeated in triplicate to ensure reliability and reproducibility of the results.

Western Blotting (WB)

Following the same protocol for cell culture and treatments, WB was done to estimate the relative abundance of the target protein. Cells were resuspended in 100 μL RIPA lysis solution with a 1% proteinase inhibitor cocktail (Roche). Following centrifugation, the total protein was collected. After loading 30 μg of total protein onto a 12% SDS-polyacrylamide gel, the protein was subjected to 80 V for 30 minutes and 120 V for 2 hours of electrophoresis. The proteins were then blocked after being transferred to PVDF membranes at 90 V for 120 minutes. After overnight incubation with the FOXN3 rabbit polyclonal antibody(Abcam, Cambridge, USA), the membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit antibodies (Zhongshan Goldenbridge Biotechnology Co. Ltd., China). GAPDH served as an endogenous control.

Time-Lapse Imaging of Live Cells

For time-lapse monitoring, melanoma cells were imaged every 1 hour using a zenCELL Owl incubator microscope (innoME GmbH, supplied by LabLogic UK) for 96 hours. Time-lapse curve were generated with zencell-owl software (version 3.3, innoME GmbH), and analysed using their built-in algorithms of relative cell coverage, proportion of detached cells, and total cell numbers. In some cases, images of cells from certain time points were further analysed in Image J (v1.52n) to cell count of rounded cells per field, and/or the proportion and length/width ratio of elongated cells.

Cell Scratch-Wound Healing Assay

Cell migration and wound-healing ability were tested using a wound-healing assay. We used transfection to alter FOXN3 expression in A375 and A875 cells. After reaching 90–95% confluence in 6-well plates, wounds were created by scratching the surface of the plates with a 10 μL pipette tip. At 0 h, the wound width will be imaged after cells have been rinsed with serum-free DMEM. The cells were subsequently cultured in 10% FBS-supplemented DMEM for 6, 12, 24 and 48 hours post-injury respectively. After that, images were again taken at predetermined intervals to measure the wound gaps, to comprehensively capture the dynamics of cell migration and wound healing. The gap widths between multiple areas were used to evaluate cell movement.

Animal Experiments and Immunohistochemistry (IHC) Staining

The study was approved by Ethics Committee of Xi'an Jiaotong University, all experiments were reviewed by the Institutional Animal Care and Use Committee on the Ethics of Animal Experiments of Xi'an Jiaotong University. Twelve female BALB/c nude mice (Beijing Vital River Laboratory Animal Technology) were randomly split into two groups in a specialized pathogen-free environment. The mice were given subcutaneous injections of either 2×10^6 A375-vector cells or 2×10^6 A375-FOXN3 cells suspended in 100 μL PBS. As a result, the size of tumor nodules was tracked every 2 days.

Meanwhile, weight, skin tone, and mobility measurements were taken to assess overall health. The formula volume = 1/2 length × width^2 was used to determine the tumor size. The mice in the control group had reduced mobility, but none perished. After 24 days, tumors were excised, measured, photographed.

Formalin-fixed, 5 μm thick sections were stained for hematoxylin and eosin (H&E) using a standard protocol. Formalin-fixed tissue samples were cut at 5 μm thickness on a cryotome and stained for anti-Ki-67 (Beijing Zsbio, P. R. China) and anti-FOXN3 (Abcam). Cut sections were fixed in acetone for one minute and incubated with primary antibody (5 μg/mL for Ki-67 antibody or 2 μg/mL for FOXN3 antibody) for 2 hours. Following this treatment, sections were incubated with the EnVison complex at 37 °C for an additional three minutes before incubation with substrate solution (DAB, 3,3'-diaminobenzidine). After each incubation, sections were washed three times with Tris Buffered saline (TBS) for 10 min each. Before counterstaining with a hematoxylin quick staining kit (Vector Labs, CA, USA), sections were washed with tap water and mounted on DAKO faramount aqueous mounting solution (DAKO, Carpinteria, CA, USA). Photomicrographs were taken on a Nikon microscope equipped with a CCD camera. Ki-67 and FOXN3 quantification was achieved by measuring the ratio of the number of cells positive for brown staining to the total cells in a given field of view at 40x magnification. Five fields of view were analyzed per section.

Immunofluorescent Staining

Cells were plated on glass coverslips and were stained for 30 minutes using anti-LC3A/B (Cat. #12741, Cell Signaling Technology, Danvers, MA), rinsed, and probed for 30 minutes using secondary AF488-anti-mouse IgG (Invitrogen). Cells were mounted using a mounting medium containing DAPI (P36935, Invitrogen) and imaged using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Different fields of view were randomly selected based on the observed DAPI signal for quantification purposes.

Establishment of Vemurafenib Resistance Cell Lines and Analyses of Cellular Proliferation

Briefly, we cultivated A375 cell line under the treatment of increasing amounts of Vemurafenib, firstly 0.5 μM Vemurafenib was used for 2 weeks, and then 2 μM Vemurafenib was added as a part of the culture medium. Cells that survive the conditions were selected and amplified.

Using a CellTiter 96® Aqueous One Solution assay (Promega), the viability of prepared melanoma cells was determined. After plating cells in 96-well plates and the supplied One Solution reagent was aliquoted into each well. Each well's absorbance at 490 nm was measured. The half-maximal inhibitory concentration (IC_{50}) of Vemurafenib was subsequently determined by normalizing cell viability to vehicle-treated control cells (100%).

Chromatin Immunoprecipitation (ChIP)

A 1% formaldehyde solution was used to cross-link A375 cells that had been transfected with Flag-tagged FOXN3 overexpression or empty control vectors (1×10^7 cell/sample) at 37 °C for 10 min, after which the Magna ChIP™ protein G Kit (Millipore Co., MA, USA) was used based on prepared directions to isolate nuclear lysates. A Covaris-S2 instrument was subsequently used to ultrasonicate these lysates, yielding 300–500 bp DNA fragments. Anti-Flag (CST) was then utilized for ChIP, with a PCR approach then being used to detect the immunoprecipitated DNA fragments using appropriate primers specific to the promoter region of interest (Supplementary Table 1).

Statistical Analyses

Data are means ± SD from a minimum of three experiments using triplicate samples. Survival analyses were performed with Log rank tests, while other data were compared using two-tailed Student's *t*-tests. $P < 0.05$ was the significance threshold.

Results

FOXN3 Expression Analysis in Human Melanoma Patients

The GEPIA, LinkedOmics, and Human Protein Atlas databases were used to probe publically available TCGA, GEO datasets, and Xiantao tools about the expression of FOXN3 in melanoma patients. GSEA approaches were also used to examine the expression of FOXN3 signaling pathway-related targets. Overall, these analyses indicated that relative to normal skin, FOXN3 expression was significantly decreased in melanoma (Figure 1A and B, and Supplementary Figure 1), whereas FSIP1 expression was increased (Figure 1C). But FSIP1 expression is not statistically significant (Figure 1C). FOXN3 expression progressively declined with the increasing progression of melanoma across different stages (Figure 1D). Bioinformatics analyses of FOXN3-related genes (Figure 1E) and KEGG analyses (Figure 1F) revealed that FOXN3 was closely associated with the TGF, Wnt and hedgehog signaling pathway. GSEA analyses reaffirmed this association between FOXN3 and Wnt signaling (Figure 1G).

FOXN3 Mediated Repression of Melanoma Cell Proliferation, Invasion, and Migration

Cell tests were conducted further to explore the potential function of FOXN3 in human melanoma. Here, we used WB to examine the expression patterns of FOXN3 protein in many different types of human cell lines, including those derived from melanoma (A875, HMY-1, SK-MEL-1, and A375 cells) and normal human cells (HaCaT cells). Protein expression levels of FOXN3 were much lower in the melanoma cell lines than in HaCaT cells, as shown in Figure 2A. For subsequent gain- and loss-of-function studies, we used A375 cells, which have the lowest endogenous FOXN3 expression, and A875 cells, which have the greatest endogenous FOXN3 expression. Next, transfection efficiency was assessed by treating cells with FOXN3 expression vectors or targeted shRNAs (shR1 and shR2), then analyzing the resulting by Western blots. Transfection of FOXN3 expression vectors dramatically increased FOXN3 protein expression compared to the control group in A375 cells (Figure 2B), whereas transfection of shR1 and shR2 significantly reduced FOXN3 expression compared to the shNC group in A875 cells (Figure 2B), notably shR1.

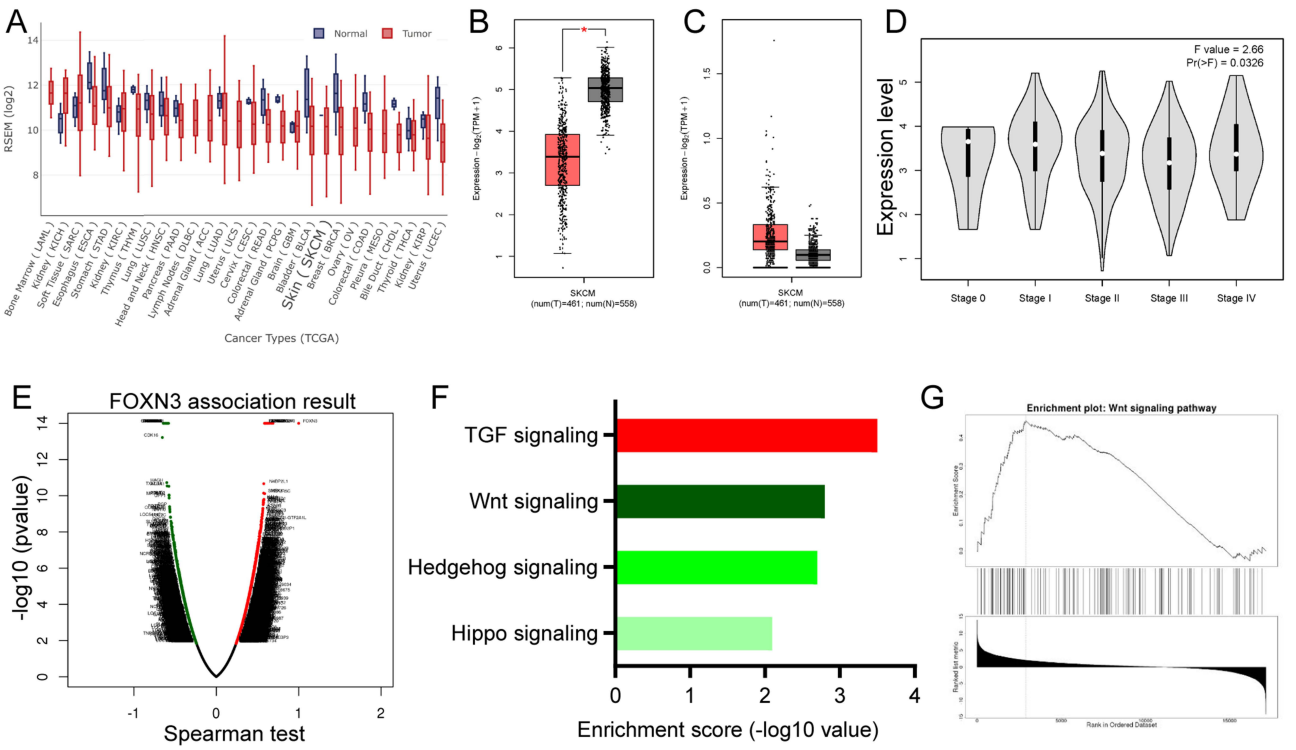

Figure 1 Bioinformatics analyses of FOXN3 expression. (**A**) The expression of FOXN3 was compared in tumor and normal tissues paired in the GEPIA database. (**B**) FOXN3 levels were compared in melanoma and normal tissue samples. (**C**) FSIP1 expression was assessed in melanoma and normal tissue samples. (**D**) Violin plot of FOXN3 by tumor stage in GEPIA website. (**E**) Analyses of correlations with FOXN3 expression in melanoma. (**F**) FOXN3 KEGG analyses. (**G**) FOXN3 GSEA analyses in melanoma.

For this reason, additional knockdown research will focus on shR1. The CCK8 test and colony formation assay showed that over-expressing FOXN3 significantly reduced the proliferation of A375 cells whereas knocking down FOXN3 dramatically increased the proliferation of A875 cells (Figure 2C and D). To assess the baseline proliferation of the cells, live cell time-lapse imaging was employed, enabling accurate evaluation of cell proliferation dynamics (zenCELL Owl). Supplementary Figure 2 illustrates the outcomes, wherein the augmented expression of FOXN3 was found to exert inhibitory effects on the proliferation of A375 cells, whereas the targeted depletion of FOXN3 exhibited a promotive impact on the proliferation of A875 cells. Furthermore, scratch assay and Transwell invasion assay demonstrated that FOXN3 over-expression treatment markedly reduced the migration and invasion abilities of A375 cells while sharply increasing the migration and invasion abilities of A875 cells in the FOXN3 depletion group compared to those in the shNC group. Notably, we found that the time point at 24 hours post-injury exhibited the most significant cell migration in A375 and A875 cell lines, emphasizing the observable changes at this crucial time point (Figure 2E and F). In contrast, the cell migration at 48 hours post-injury did not exhibit a more pronounced alteration compared to 24 hours post-injury. Collectively, our findings suggest that FOXN3 inhibits the growth, migration, and invasion of melanoma cells.

FOXN3 Over-Expression Mediated in vivo Growth Retardation in Melanoma Cells

As a follow-up to the in vitro results, tumor transplantation models were produced in mice by subcutaneously injecting empty vector or FOXN3 expression vector-treated A375 cells into the animals' flanks. Tumor growth in the FOXN3 over-expression group was significantly slower than in the control group, as seen in Figure 3A and B. The tumors removed from the FOXN3 over-expression group were also noticeably lighter than those from the control group (Figure 3C). Additionally, tumors from nude mice were immunostained to reveal the patterns of expression of the Ki67 and FOXN3 proteins. Tumors with FOXN3 over-expression had a significantly lower percentage of Ki67-positive cells compared to tumors without over-expression (Figure 3D). Further, there were significantly more FOXN3-positive

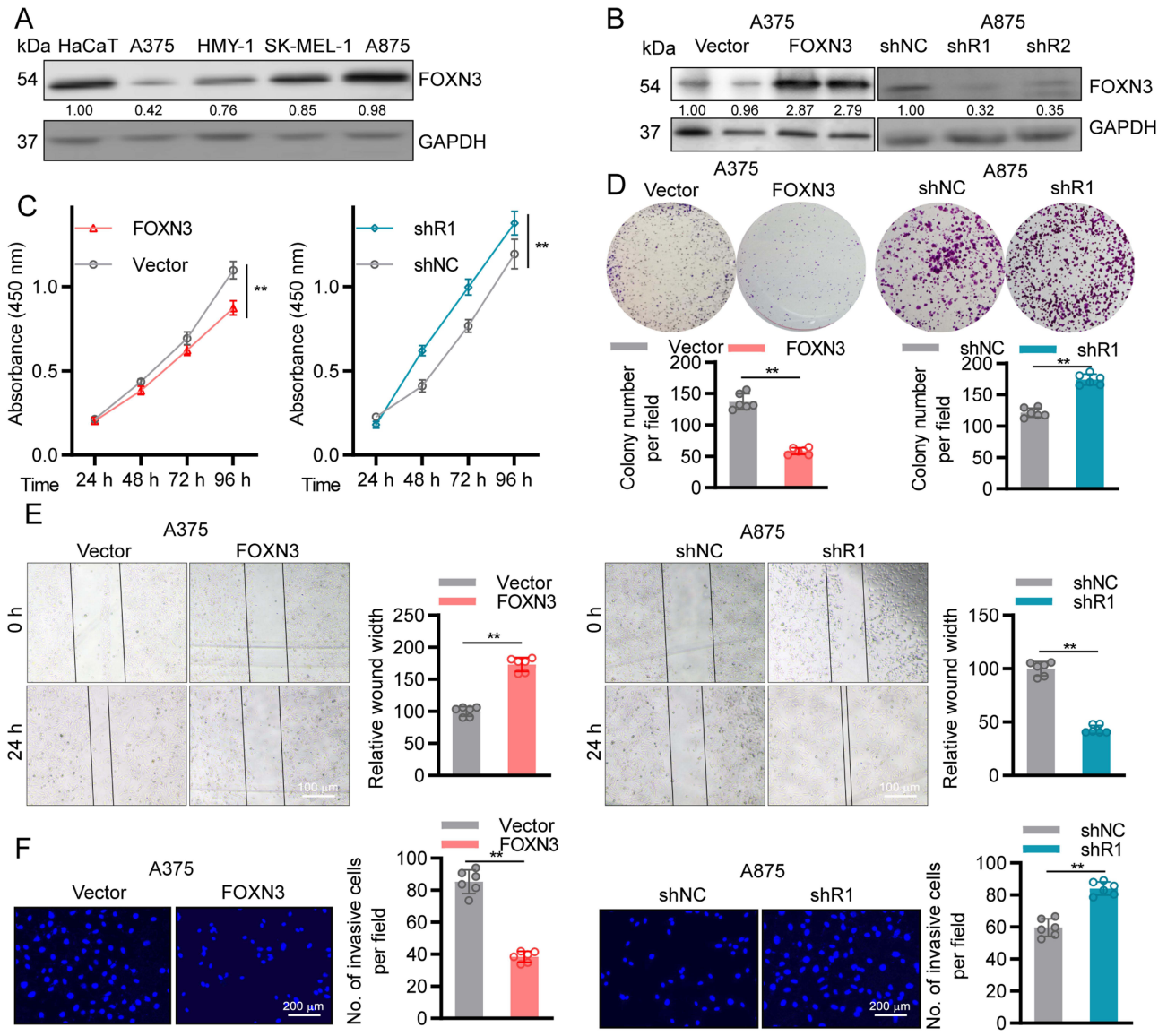

Figure 2 FOXN3 mediated suppression of melanoma cell proliferation, migration, and invasion. (**A**) Western blotting analyzed the amount of FOXN3 protein expression in normal human HaCaT cells and four different melanoma cell lines (A375, HMY-1, SK-MEL-1, and A875). (**B**) After transfecting A375 cells with FOXN3 expression vectors or A875 cells with particular shRNAs, transfection efficiency was determined by WB. (**C**) After transfecting A375 cells with FOXN3 expression vectors and A875 cells with shR1, cell proliferation was assessed using CCK8 assays. (**D**) The transfection of FOXN3 expression vectors into A375 cells or the transfection of shR1 into A875 cells was followed by colony formation assays to evaluate their capacity to form colonies. (**E**) Scratch wound healing assays were used to evaluate migratory potential after transfection of A375 cells with FOXN3 expression vectors or transfection of A875 cells with shR1. (**F**) Transwell invasion assays using DAPI labeling were used to evaluate invasive potential after transfection of A375 cells with FOXN3 expression vectors or A875 cells with shR1. $^{**}P < 0.01$.

cells in the FOXN3 over-expression group compared to the control group (Figure 3D). Consistent with in vitro results, our data show that over-expression of FOXN3 inhibits melanoma cell proliferation in vivo.

FOXN3 Localization Analysis in Vemurafenib-Resistant and Sensitive Melanoma Cells

Increasingly studies have revealed that changes in cell autophagy are related to drug resistance.[25] Thus, we identified autophagy through immunofluorescent. Immunofluorescent staining revealed a significant increase in cytoplasmic autophagosome numbers in Vemurafenib-resistant melanoma cells (A375R) relative to drug-sensitive parental cells (A375) (Figure 4A). Western blotting analysis also showed an increased expression of LC3I/II protein in A357R compared to A375. (Figure 4B). We utilized transmission electron microscopy (TEM) to examine autophagosomes and conducted a quantitative analysis. The results show that there are more autophagosomes in A375R cells (Figure 4C).

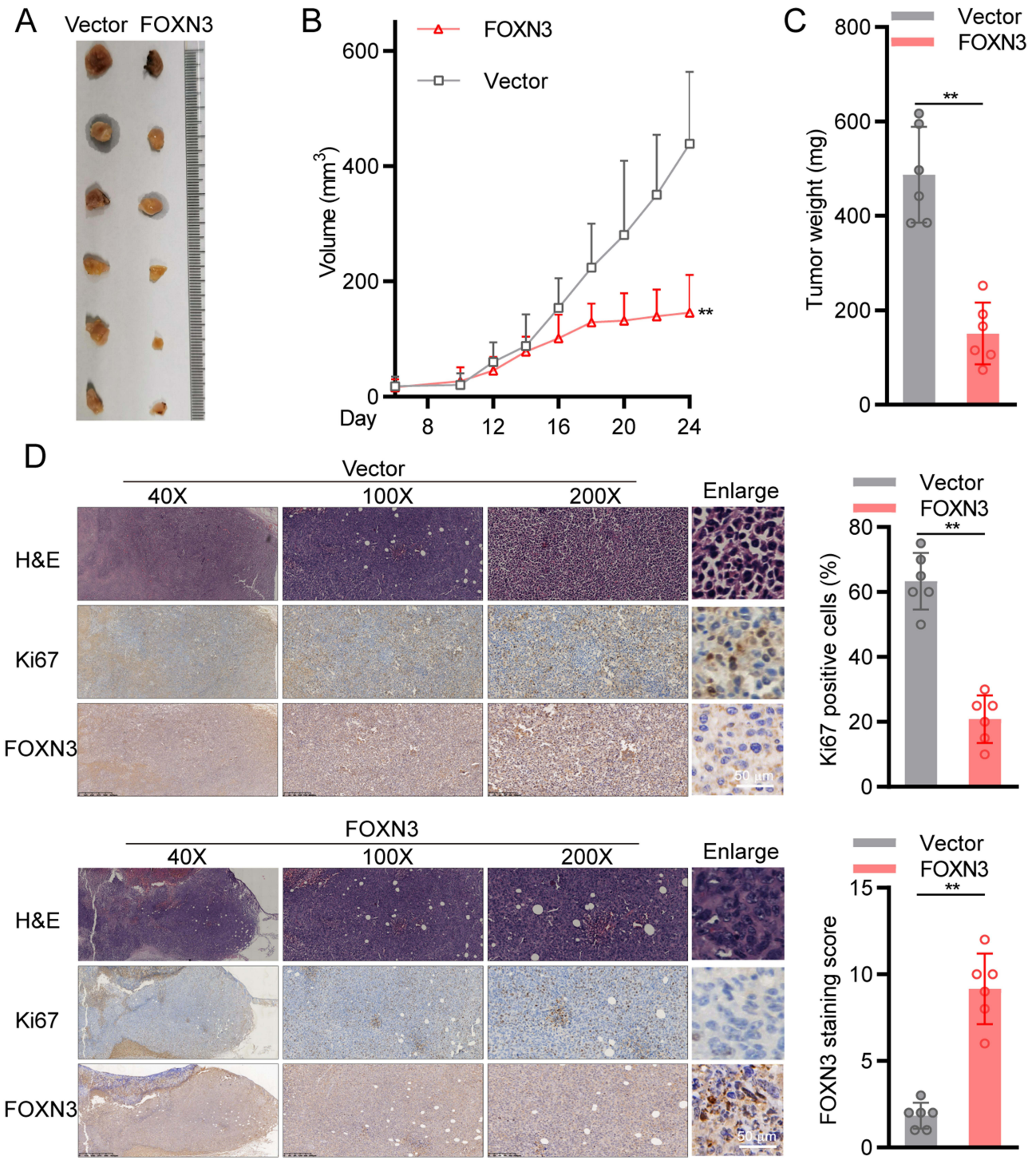

Figure 3 FOXN3 over-expression mediated retardation in melanoma cell growth in vivo. (**A**) Representative images of tumors harvested from melanoma xenograft models. (**B**) Based on the formula volume (mm^3) = [length (mm) x $width^2$ (mm^2)] / 2, a growth curve for the tumor was generated. (**C**) On day 24 post-transplantation, tumor samples were taken and weighed. (**D**) Immunohistochemical labeling revealed protein expression patterns for Ki67 and FOXN3 in tumors extracted from nude mice. $^{**}P < 0.01$.

Next, Vemurafenib-sensitive and -resistant A375 cells were transfected with a vector to overexpress FOXN3. In drug-sensitive A375 cells, FOXN3 was primarily localized to the cytosol, whereas in A375R cells, it was primarily localized to the nucleus, with significantly reduced cytoplasmic FOXN3 levels in these drug-resistant cells (Figure 4D and E). It is known that FOXN3 acts as a transcription factor, and its localization to the nucleus is important for its function to drive

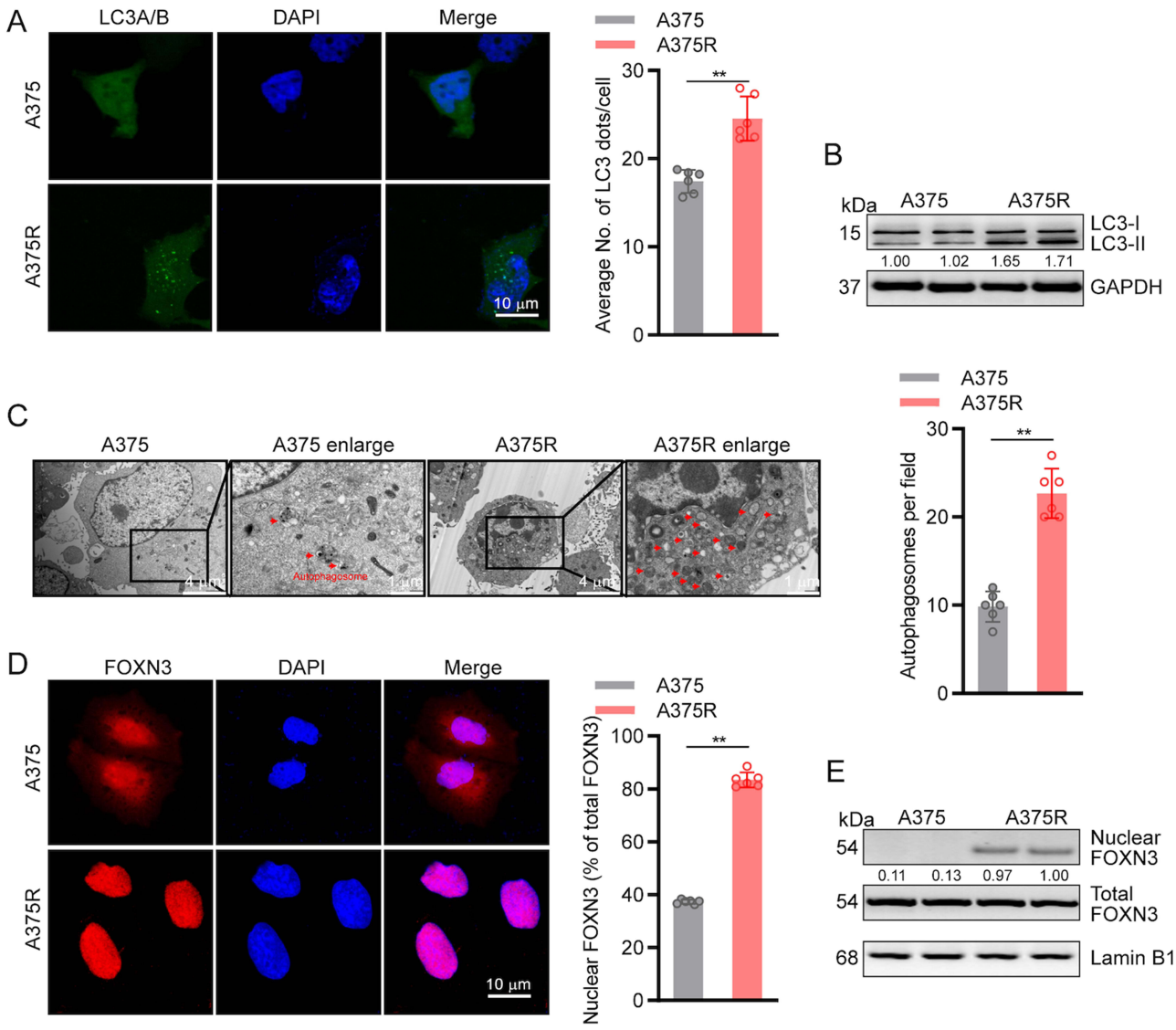

Figure 4 Autophagic activity analysis detection via immunofluorescent staining in melanoma cells. (**A**) Significantly more autophagosomes were evident in drug-resistant cells as compared to drug-sensitive cells. Scale bar: 10 μm. (**B**) Western blotting analyzed the amount of LC3I/II protein in melanoma cell lines A357 and A375R. (**C**) TEM images of autophagosomes in A375 and A375R cells. Red arrows enote autophagosomes. The numbers of autophagic vacuoles were determined by quantitative analysis. Scale bar: 4 μm, enlarge scale bar: 1 μm. (**D**) Differences observed in FOXN3 localization in drug-sensitive and drug-resistant melanoma cells via immunofluorescent staining. In Vemurafenib-sensitive melanoma cells, FOXN3 primarily localized to the cytosol, whereas in Vemurafenib-resistant cells, it was primarily found in the nucleus. Scale bar: 10 μm. (**E**) Western blotting analyzed the nuclear and total FOXN3 expression in melanoma cell lines A357 and A375R. Lamin B1 was loading as the nuclear control. $^{**}P < 0.01$.

the expression of downstream proteins and regulate drug resistance in this cell line. Therefore, we believe that FOXN3 localization to the nucleus plays a crucial role in mediating the drug resistance in A375R cells.

FOXN3 Over-Expression Mitigates Vemurafenib Resistance and Suppressed Autophage in A375R Cells

Next, FOXN3 was overexpressed in A375R cells, and changes in Vemurafenib resistance were evaluated; a significant decrease in the measured IC_{50} value was observed (Figure 5A). The overexpression of FOXN3 in A375R cells led to a notable reduction in the measured IC50 value for Vemurafenib, indicating a heightened sensitivity to the drug. Additionally, LC3A/B immunofluorescence staining demonstrated that FOXN3 overexpression significantly inhibited autophagy in these cells (Figure 5B). In our investigation, wherein we sought to elucidate the impact of FOXN3

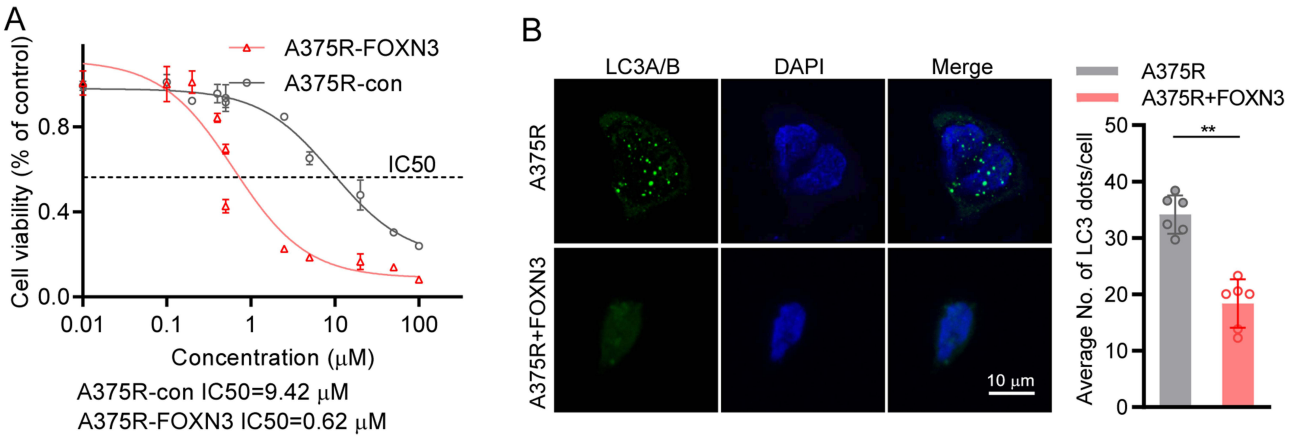

Figure 5 The impact of FOXN3 on A375 cells resistance to drug treatment and autophagic activity. (**A**) The impact of FOXN3 on drug IC_{50} values. (**B**) The impact of FOXN3 on A375R cell autophagy. Scale bar: 10 μm. $^{**}P < 0.01$.

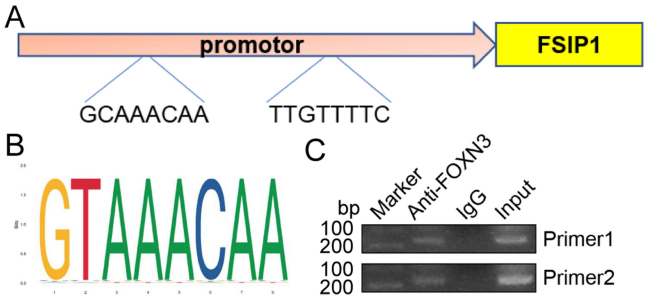

Figure 6 Confirmation of the FOXN3 ability to bind the FSIP1 promoter. (**A** and **B**) The JASPAR software was used to predict putative sites of FOXN3 binding within the region upstream of the *FSIP1* start codon. (**C**) DNA isolated via ChIP was utilized as a template for PCR analyses, with initial amplification performed with primer 1 followed by the separation of amplified products via agarose gel electrophoresis, with subsequent repeat amplification with primer 2.

overexpression in A375 cells, we regrettably failed to detect any discernible alterations in the levels of autophagy (Supplementary Figure 3). The present results highlight the intricate nature of autophagy regulation in diverse cellular contexts, shedding light on the nuanced intricacies that govern this cellular process in a cell line-dependent manner. The observed inhibition of autophagy, as evidenced by LC3A/B immunofluorescence staining, suggests a potential mechanistic link between FOXN3 expression and autophagic processes in the context of Vemurafenib resistance. These findings FOXN3 as a pivotal regulator influencing both Vemurafenib resistance and autophagy pathways, contributing valuable insights for further exploration in melanoma treatment strategies.

Identification of FOXN3 Binding Sites Within the FSIP1 Promoter

Bioinformatics tools identified two putative sites of FOXN3 binding upstream of the *FSIP1* start codon (ATG) located at −617 to −624 bp and −1267 to −1274 bp (Figure 6A and B). The subsequent ChIP experiments confirmed the ability of FOXN3 to bind to these sites in A375R cells (Figure 6C). This discovery sheds light on the molecular interactions between FOXN3 and FSIP1, presenting a foundation for further exploration into the regulatory roles and functional consequences of this binding in the context of melanoma and drug resistance.

Discussion

In this study, we revealed a significant decrease in FOXN3 expression in melanoma tissue compared to normal skin tissue samples, with this expression closely correlated with the activity of the Wnt signaling pathway. Immunofluorescent staining revealed increased autophagic activity in drug-resistant melanoma cells, and FOXN3 was found to preferentially

localize to distinct cell compartments in response to Vemurafenib resistance. Overexpression of FOXN3 was sufficient to reduce autophagic activity significantly. FOXN3 was discovered to bind the FSIP1 promoter, thereby regulating the expression of this gene. These findings suggest that FOXN3 may be an attractive therapeutic intervention target for overcoming melanoma tumor drug resistance.

According to a previous study, while approximately 80% of patients with BRAF-mutated melanoma respond to Vemurafenib when initially treated, they typically develop drug resistance within one year.[26] FOXN3 was found to increase drug-resistant melanoma cells' sensitivity to Vemurafenib. Overexpression of FOXN3 decreased the IC_{50} value of Vemurafenib in these cells, suggesting that this transcriptional regulator may play a mechanistic role in drug resistance.

The development of BRAF-specific MEK inhibitors and immunotherapeutic drugs offered novel treatment options to patients suffering from melanoma.[27] The RAF family serine/threonine protein BRAF functions as an upstream regulator of MAPK and MEK signaling and downstream of RAS, controlling the ability of cells to proliferate in response to diverse growth signaling mediators under physiological conditions.[28] Activating NRAS mutations are present in ~28% of melanoma cases, while BRAF mutations are present in ~52% of melanoma cases, with the V600E mutation being the most common such alteration.[29]

Chemotherapeutic drugs are used as standard treatments for various cancer types, but antitumor drug resistance is a common clinical finding associated with treatment failure.[5] Drug resistance includes both intrinsic and acquired resistance cases, and numerous mechanisms have been linked to this resistance activity, including changes in drug metabolism, epigenetic alterations, drug target mutations, shifts in tumor microenvironment composition, and/or altered drug efflux.[30] Given their ability to influence processes involving growth factors, angiogenesis, and degradation of the extracellular matrix, members of the FOX family of transcriptional regulators may serve as important mediators of drug resistance. FOXA1 has been reported to play a role in resistance against tamoxifen in ER-positive breast cancer cases, while FOXD1 targets p27 in breast cancer to enhance resistance to chemotherapeutic drugs,[31] and FOXD3 is reportedly upregulated in melanoma cells harboring BRAF mutations resistant to treatment with PLX4032 and PLX4720.[32] FOXD3 is upregulated in an adaptive response following treatment with RAF inhibitor drugs that contribute to resistance against these agents.[32] By targeting and suppressing the transcription of FOXC2, FOXF2 can inhibit multidrug resistance in BLBC cells.[33] Efforts to target FOXM1 can enhance platinum-resistant ovarian cancer cell sensitivity to cisplatin and paclitaxel-mediated killing.[34] FOXM1 can promote ER-positive breast cancer cell invasivity and endocrine resistance, facilitating stem-like tumor cell expansion.[35]

Prior work has indicated that FOXN3 is an essential mediator of the onset and progression of various cancers. Xue et al found FOXN3 to be downregulated in osteosarcoma, with such downregulation regulating SIRT6 expression to control the invasivity and migration of the tumor cells.[36] Researchers also observed a marked drop in the expression of FOXN3 in acute myeloid leukemia, with FOXN3 suppressing tumor growth through the induction of apoptosis and the suppression of proliferative activity.[20] Zhao et al determined that papillary thyroid cancer was associated with a reduction in FOXN3 expression, with this transcription factor serving to inhibit invasivity and growth by suppressing the Wnt/β-catenin pathway.[19] Chen et al and Sun et al also reported the ability of FOXN3 to suppress proliferative activity, metastasis, and EMT induction in tongue squamous cell carcinoma and melanoma.[37,38] We revealed that FOXN3 participated in drug resistance by regulating autophagy activity in melanoma.

In addition to the comprehensive alignment of our findings with the study's objectives and the broader context of melanoma drug resistance research, further exploration of the obtained data reveals intriguing avenues for future investigation. Specifically, delving deeper into the molecular mechanisms by which FOXN3 regulates autophagic activity in drug-resistant melanoma cells could uncover novel therapeutic targets and strategies for combating resistance. Additionally, elucidating the interplay between FOXN3 and other signaling pathways implicated in melanoma progression and resistance could provide a more comprehensive understanding of the intricate network of factors contributing to treatment failure. Furthermore, investigating the potential crosstalk between FOXN3 and other transcriptional regulators known to modulate drug resistance may unveil synergistic or antagonistic interactions that could be leveraged for therapeutic benefit. Overall, by expanding our focus on the obtained data and its implications, we can uncover new

insights that advance our understanding of melanoma resistance mechanisms and inform the development of more effective treatment approaches.

Conclusion

In conclusion, our study elucidated the critical role of FOXN3 in melanoma progression and drug resistance. Our findings demonstrated that FOXN3 is downregulated in melanoma tissues and acts as a potent inhibitor of melanoma cell proliferation and motility. Importantly, we showed that FOXN3 overexpression reduces autophagic activity in melanoma cells, implicating FOXN3 in the regulation of this crucial cellular process. Furthermore, our results revealed significant differences in FOXN3 localization between drug-sensitive and resistant melanoma cells, suggesting its involvement in mediating drug resistance mechanisms. Additionally, our study identified FOXN3's interaction with the promoter region of FSIP1, indicating its regulatory role in melanoma development and drug resistance. Through these findings, we proposed FOXN3 as a promising therapeutic target for overcoming drug resistance in melanoma patients. By targeting FOXN3, we may potentially disrupt key pathways driving resistance, leading to improved treatment outcomes for individuals suffering from drug-resistant melanoma. Overall, our study underscores the importance of FOXN3 in melanoma biology and highlights its potential as a valuable target for future therapeutic interventions.

Data Sharing Statement

We declare that the bioinformatics analysis data provided is based on the public database provided by the GEPIA database (http://gepia.cancer-pku.cn/detail.php) and the LinkedOmics database (http://www.linkedomics.orglogin.php).

Ethics Statement

Our study was approved by the Ethics Committee of Medical Department of Xi'an Jiaotong University, No. 2020-934.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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