Comprehensive Investigation into the Role of Ubiquitin-Conjugating Enzyme E2S in Melanoma Development

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Ubiquitin-conjugating enzyme E2S (UBE2S) is involved in protein degradation and signal transduction, but its function in the development of melanoma is unclear. We focused on the role of UBE2S in melanoma development both in vitro and in vivo. UBE2S was overexpressed in malignant melanoma cells and tissues, and UBE2S expression was significantly different between tumor node metastasis staging T4 and T1/T2/T3. We designed UBE2S short hairpin RNA (shUBE2S) and transfected it into A375, SK-MEL-28, and MUM-2B cells using lentivirus. By whole-genome filtering, 247 genes and 265 genes were upregulated and downregulated, respectively, in shUBE2S-treated melanoma; these genes were mainly involved in immune reactions, apoptosis, DNA damage repair, and cell movement. The proliferation of melanoma cells was inhibited, apoptosis was increased, and cell cycle was arrested in G1/S in shUBE2S-treated melanoma. Expression of epithelial to mesenchymal transition–related proteins was significantly suppressed, and tumor growth was also suppressed in shUBE2S BALB/C nude mice. shUBE2S treatment may cause cell cycle arrest in G1/S phase, inhibit proliferation, induce apoptosis, and suppress tumor growth through DNA damage repair, epithelial to mesenchymal transition inhibition, protein kinase B-mTOR pathway, NF-κB signaling, and immune reactions, which provides a comprehensive understanding of the role of UBE2S in melanoma development and the need for advanced clinical research into UBE2S.

INTRODUCTION

Melanoma is an aggressive and severe form of skin cancer caused by malignant melanocyte proliferation, and the prevalence of this cancer is increasing more rapidly than any other form of cancer. (Balch et al., 2009; Leonardi et al., 2018; Lo and Fisher, 2014; Siegel et al., 2019). Exposure to UVR from sunlight and other UVB spectrum sources is the main environmental risk factor for cutaneous melanoma development (Gichrest et al., 1999; Leonardi et al., 2018), although host risks factors such as family history and genetic mutations play a crucial role in melanoma development as well (Goldstein and Tucker, 2001; Leonardi et al., 2018; Soura et al., 2016). BRAF, NF1, and NRAS mutations are frequent genetic drivers in the melanomas associated with melanocyte overproliferation, abnormal metabolism, apoptosis resistance, and cell cycle arrest, which lead to the aberrant activation of the RAS/RAF/MAPK/extracellular signal–regulated kinase (ERK) (MEK)/ERK signaling cascade (MAPK pathway) and the phosphoinositol-3-kinase/protein kinase B (Akt) pathway (Chappell et al., 2011). In recent years, targeted therapy, such as the use of RAF and MEK inhibitors, have been extensively used in the clinical treatment of melanoma (Mishra et al., 2018). Immune checkpoint inhibitors, including anti-CTLA4 and anti–PD1 and/or PD-L1, have also become common in melanoma treatment. Unfortunately, drug resistance to BRAF/MEK inhibitors inevitably emerge within 6–12 months of treatment initiation, and relapses are associated with downstream activation of the MAPK pathway and the parallel signaling pathways (phosphoinositol-3-kinase/AKT) (Flaherty et al., 2012). For most patients with metastatic melanoma, the long-term prognosis remains poor, even with the use of novel therapeutic methods.

Ubiquitination and deubiquitination are crucial post-translational modifications involved in the pathogenesis of melanoma development through key signaling pathways (Ma et al., 2017). For example, ubiquitin-conjugating enzyme E2N promotes melanoma growth through MEK/FRA1/STX10 signaling (Dikshit et al., 2018), USP28 and/or FBW7 suppresses the MAPK pathway by targeting BRAF for degradation (Saei and Eichhorn, 2018), and FBXO38 is an E3 ligase of PD-1–mediating Lys48-linked poly-ubiquitination and degradation, which regulates PD-1 expression and provides a

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Abbreviations: AKT, protein kinase B; EMT, epithelial–mesenchymal transition; ERK, extracellular signal–regulated kinase; IPA, ingenuity pathway analysis; LXR, liver X receptor; MEK, MAPK/ERK kinase; RXR, retinoid X receptor; shCtrl, short hairpin RNA control cell; shUBE2S, UBE2S short hairpin RNA; UBE2S, ubiquitin-conjugating enzyme E2S

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Potential block for the PD-1 pathway (Meng et al., 2018). Deubiquitinase ubiquitin-specific protease 4 regulates cisplatin-induced apoptosis by p53 signaling and promotes the invasive and migratory behaviors of melanoma through epithelial–mesenchymal transition (EMT) (Hoeller and Dikic, 2009). Ubiquitylation regulates crucial pathways in the melanoma pathogenesis, including the NF-κB pathway MITF and cell cycle (Ma et al., 2017).

Ubiquitylation-related proteases, which include E2 conjugating enzyme and E3 ligase, play an important role in the protein degradation and signal transduction involved in the development of melanoma (Hoeller and Dikic, 2009; Miller and Mihm, 2006; Nakayama, 2010). Ubiquitin-conjugating enzyme E2S (UBE2S) is overexpressed in several cancers, in which it is associated with cancer growth and G2/M phase arrest, such as cervical cancer, oral squamous cell carcinoma, and breast cancer (Yoshimura et al., 2017). The E2S family contains approximately 40 members with a highly conserved 150 amino acid core domain, and the family members are involved in inflammation, cell cycle regulation, and cancer metastasis (Chen and Chen, 2013; Gallo et al., 2017). Aberrant regulation of UBE2S expression influences signaling of inflammatory NF-κB and TGFβ, mitogenic GFS, and hypoxia-inducible factor transcription factors (Gallo et al., 2017), although the role of UBE2S in the development of melanoma has not yet been elucidated.

In this study, we designed a UBE2S short hairpin RNA (shUBE2S) and used a lentivirus to deliver it to three melanoma cell lines, which was followed by a series of tests of cell proliferation, apoptosis, and cell cycle processes. We determined the pathways and networks that were involved through genome expression microarray. Inhibition of tumor growth in vivo was validated using shUBE2S-treated BALB/C nude mice. Through summarizing the custom networks and discussing the interacting pathways, we demonstrated that UBE2S plays an important role in the development of melanoma.

Effective UBE2S knockdown after shUBE2S transfection
Transfected A375, SK-MEL-28, and MUM-2B cells were analyzed by fluorescence microscopy 72 hours after lentivirus transfection and found to have a transfection efficiency of over 80% (Supplementary Figures S1a–d, S2a–d, and S3a–d). UBE2S was downregulated in short hairpin RNA UBE2S-transfected A375, SK-MEL-28, and MUM-2B cells (shUBE2S) compared with short hairpin RNA control cells (shCtrls) (Supplementary Figures S1e, S2e, and S3e). UBE2S expression was significantly inhibited in shUBE2S, and the efficiency of A375, SK-MEL-28, and MUM-2B UBE2S knockdown was over 68%, 65%, and 45%, respectively. FLAG decreased in shUBE2S versus shCtrl, demonstrating that the designed shUBE2S effectively resulted in knockdown and that it was an effective system (Supplementary Figures S1f, S2f, and S3f).

Differentially expressed genes were analyzed and validated
Total RNA of the transfected melanoma cells reached the quality requirement for microarray assay. The genome expression microarray results (Gene Expression Omnibus accession: GSE115922) showed that 247 genes were upregulated and 265 genes were downregulated by over two-folds in the shUBE2S group when compared with the shCtrl (Supplementary Table S4), whereas UBE2S (ID27338) was downregulated by over four-folds in the shUBE2S group. A histogram of the expression illustrated the distribution of all the microarray probes (Figure 1c). All signal curves were highly coincident within an overlap, which showed that all microarray results were reliable (Figure 1c). The relative log expression signal box includes all of the log distributions of the microarray data, and the upper and/or lower horizontal lines are the 90% confidence intervals (Figure 1d). The similar distribution of the relative expression box proved the high repeatability of all the samples. The volcano plot displayed the distribution of differently expressed genes between shUBE2S and shCtrl, with the most differentially expressed genes located in the top right and/or left corners (Figure 1e). The heatmap graph displayed the clustering of differently expressed genes, and there was a high within-group correlation (Figure 1f).

We selected 27 differentially expressed genes for validation using qPCR. The PCR primers are listed in Supplementary Table SM2 (Supplementary Materials and Methods). FOS, STAT1, EGR1, IRF9, CLU, IFIT1, IFIT3, IFITM1, C3, IFI35, IFI6, ISG15, OAS1, NEDD4, MX1, and MLYL10 genes were upregulated in the shUBE2S group compared with the shCtrl group (Figure 1g), whereas MYC, CCL2, MMP9, PTGS2, TNFRSF11B, ETS1, TNFAIP3, LPL, IL36G, SCD, and CLDN1 genes were downregulated in the shUBE2S group (Figure 1g). The gene expression tendency was highly consistent with the microarray results, which validated the reliability of those microarray results (Supplementary Table S4).

Analysis of the involved pathways and networks
Canonical pathways analysis. Canonical signaling pathways involved in the differently expressed genes were analyzed using ingenuity pathway analysis (IPA). The z-score > 2 indicated a significantly active pathway and z-score < −2 indicated an inhibited pathway. IFN signaling
**Figure 1.** Protein expression of UBE2S in tissues and gene expression microarray profiling of shUBE2S-treated A375 melanoma cells. (a) Image of melanoma tissue array subjected to immunohistochemistry. Each sample (serial number) was listed from top left to bottom right, with 16 samples for each row. Sample numbers of 193–208 were nonmelanoma skin tissues. Bar = 2 mm. (b) The UBE2S protein expression in malignant melanoma and nonmelanoma tissues. (c) Expression histogram of all GeneChip probes. The abscissa indicates the interval of the probe expression, whereas the ordinate indicates the probe statistic quantity within the interval; each curve corresponds to one sample. (d) Relative signal box plot. The abscissa indicates each sample, and the ordinate indicates the relative log expression value. The red line in the middle indicates the mean value of the relative log value of all samples. (e) Volcano plot graph of all probes. The abscissa indicates the log-transferred fold change with two as the base number, and the ordinate indicates the log-transferred corrected significant level with 10 as the base number. Red dots correspond to differentially expressed genes with a greater than two-fold change ($P < 0.05$). (f) Heatmap of differentially
were upregulated in the shUBE2S group, which correlated (Supplementary Table S5). The directly interactive relationship between the network (Supplementary Figure S4). A total of 32 genes were downregulated through differently expressed genes clustered by their expression profiling. Red indicates upregulated genes, green indicates downregulated genes, black indicates middle values, and gray indicates nondetected values. The upper cluster refers to all samples, and the left indicates each gene. Expression validation of the 27 selected differently expressed genes in shUBE2S using qPCR. *P < 0.05; **P < 0.001. UBE2S, ubiquitin-conjugating enzyme E2S; RMA, Robust Multiarray Average; shCtrl, short hairpin RNA control cell; shUBE2S, UBE2S short hairpin RNA.

Table 1. Canonical Pathways Analysis and Involved Genes

<table>
<thead>
<tr>
<th>Ingentiva Canonical Pathways</th>
<th>(-\log (P\text{-value}))</th>
<th>Z-Score</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN signaling</td>
<td>8.52</td>
<td>3</td>
<td>IFIT3, IFIT1, OAS1, MX1, IFI35, IFI6, IRF9, STAT1, IFITM1, and ISG15</td>
</tr>
<tr>
<td>LXR/RXR activation</td>
<td>2.91</td>
<td>2.65</td>
<td>SCD, IL36G, C3, CCL2, LPL, PTGS2, MMP9, CLU, TNFRSF11B</td>
</tr>
<tr>
<td>Role of pattern recognition Receptors in recognition of bacteria and viruses</td>
<td>3.11</td>
<td>2</td>
<td>PTX3, IFIH1, OAS1, IRF7, IL12A, C3, OAS2, DDX58, RNASE1, IL11</td>
</tr>
<tr>
<td>Neuropathic pain signaling in dorsal horn neurons</td>
<td>0.636</td>
<td>2</td>
<td>FOS, GRM8, PLCB1, TAC1</td>
</tr>
<tr>
<td>RhoA signaling</td>
<td>0.37</td>
<td>2</td>
<td>NEDD4, MYL10, CDC42EP3, PPP1CB</td>
</tr>
<tr>
<td>Production of nitric oxide and reactive oxygen species in macrophages</td>
<td>0.624</td>
<td>1.63</td>
<td>FOS, PPP1CB, STAT1, PPP2KB, CLU, TNFRSF11B</td>
</tr>
<tr>
<td>Signalining by Rho family GTPases</td>
<td>0.547</td>
<td>1.63</td>
<td>FOS, NEDD4, CDH12, MYL10, GNG2, WASP3, CDC42EP3</td>
</tr>
<tr>
<td>PPAR signaling</td>
<td>1.3</td>
<td>1.34</td>
<td>FOS, NDS3, NRAS, PTGS2, TNFRSF11B</td>
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<tr>
<td>CXCR4 signaling</td>
<td>0.834</td>
<td>1.34</td>
<td>FOS, NRAS, MYL10, EGR1, GNG2, PLCB1</td>
</tr>
<tr>
<td>Role of NFAT in cardiac hypertrophy</td>
<td>0.4</td>
<td>1.34</td>
<td>NRAS, GNG2, PLCB1, MEF2C, IL11</td>
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<tr>
<td>IL-8 signaling</td>
<td>0.872</td>
<td>1.13</td>
<td>FOS, NRAS, GNG2, CXCL1, PTGS2, MMP9, IRAK2</td>
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<tr>
<td>ERK2 signaling</td>
<td>1.3</td>
<td>1.34</td>
<td>MYC, RPL27A, NRAS, RPS20, RPL22L1, PAP1, PPP1CB, RPS15A, RPS2</td>
</tr>
<tr>
<td>NF-kB signaling</td>
<td>0.46</td>
<td>1.34</td>
<td>TRAF3, IL36G, NRAS, TNFAIP3, TNFRSF11B</td>
</tr>
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</table>

Abbreviations: GTP, guanosine triphosphate; LXR, liver X receptor; NFAT, nuclear factor of activated T cell; RXR, retinoid X receptor.

was found to be the significantly activated (z-score = 3) pathway. A total of 10 differentially expressed genes that were upregulated in the shUBE2S group, IFIT3, IFIT1, OAS1, MX1, IFI35, IFI6, IRF9, STAT1, IFITM1, and ISG15, were involved in this pathway (Table 1). The liver X receptor (LXR)/retinoid X receptor (RXR) activation pathway was also activated, and nine genes were involved in this signaling (Table 1). The genes of complement C3 (C3) and clusterin (CLU) were upregulated, and SCD, IL36G, CCL2, LPL, PTGS2, MMP9, and TNFRSF11B were downregulated in the shUBE2S group. NF-kB signaling was inhibited and involved TRAF3, IL36G, NRAS, TNFAIP3, and TNFRSF11B gene downregulation in the shUBE2S group (Table 1). The NRAS gene was downregulated in the shUBE2S group (Supplementary Table S4); through IPA canonical pathway analysis, ERK5 signaling was also found to be downregulated through differently expressed MYC, FOS, NRAS, RPS6KA3, and MEF2C (z-score = -0.447).

The upstream regulators of differently expressed genes (e.g., transcription factors, small RNA, receptors, and kinase) were analyzed by IPA. Among these regulators, IFNA2 was found to be a strongly activated upstream regulator that targeted 38 genes in the microarray dataset (Supplementary Table S5). The directly interactive relationship between IFNA2 and its target genes in the dataset were displayed in the network (Supplementary Figure S4). A total of 32 genes were upregulated in the shUBE2S group, which correlated with active IFNA2 status (Supplementary Figure S4). NF-kB (complex) was predicted to be a strongly inhibited upstream regulator that targeted 58 genes in the dataset (Supplementary Table S5); 42 of those genes were downregulated in the shUBE2S group. We also found that the protein NF-kB-p65S was downregulated in the shUBE2S group (Figure 2).

Function and disease analysis. The involved diseases and function enrichments were analyzed and displayed in Supplementary Table S6. All involved diseases and functions were sorted according to the activation z-score. The functions of cell death, necrosis, apoptosis, and inflammatory demyelinating disease were activated. Through IPA analysis, 176 differentially expressed genes were found to be involved in cell death and survival. The cell movement, cell migration, tumorigenesis and/or neoplasia of the tissue, and the accumulation of phagocytes and/or neutrophils and/or granulocytes and/or myeloid cells was significantly inhibited in the shUBE2S group (Supplementary Table S6). A total of 32 differentially expressed genes were involved in cell movement, and most notably, 390 genes were involved in the function of tumorigenesis and/or neoplasia of tissue.

Interaction network. Regulation effective analysis displayed the interactive relationships between regulators and differently expressed genes as well as the involved functions. The consistency score was a criterion for evaluating the causality, consistency, and connection of the upstream regulators in the network, datasets, diseases, and functions. The top regulation network showed that the regulators (e.g., ACKR2, BTK, and CNOT7) activated inflammatory demyelinating disease, neuromuscular disease, and progressive motor neuropathy and inhibited the replication of Hepatitis C virus through A2M, ABLIM3, AHCY, and other genes (Supplementary Figure S5).

Function networks displayed the activated and inhibited relationships between the genes and functions. Networks were divided into several subgroups by the IPA network generator method. On the basis of hypergeometric distribution, scores were acquired for each network. All networks were sorted according to score value, and the top network

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mainly covered RNA posttranscriptional modification, protein synthesis, DNA replication, recombination, and repair, in which 31 genes were involved. The network diagram showed the functions and diseases involved in the dataset and the predicted relationship of upregulation and downregulation using the Ingenuity Knowledge Database (Figure 3a). The other networks involved were inflammatory response, cellular development, cellular growth and proliferation, and dermatological diseases and conditions.

Customized networks were drawn on the basis of the involved pathways, upstream regulators, diseases, and function networks by IPA. The related genes in the network contained 11 differently expressed genes that originated from the involved pathways and 9 target genes (Figure 3b). FOS, IFI35, IFI6, IFIT1, IFIT3, IFITM1, IL11, IRF9, ISG15, MX1, OAS1, RUNX2, and STAT1 were upregulated in the shUBE2S group, whereas MMP9, TNFRSF11B, and UBE2S were downregulated (Supplementary Table S4). The change in UBE2S regulated STAT1, MMP9, RUNX2, IRF9, ISG15, and FOS through TP53 inhibition (Figure 3b), and UBE2S also influenced IFITM1, MMP9, RUNX2, FOS, and STAT1 through CDH1 and EGFR (Figure 3b). We validated the related proteins in melanoma cells by western blot. ISG15 and STAT1 proteins were upregulated in the shUBE2S group (Figure 2), whereas TNFRSF11B, MMP9, p-AKT, mTOR, and MYC proteins were downregulated (Figure 2). The shUBE2S treatment may have activated IFN signaling and LXR/RXR activation, and it may have inhibited the NF-κB pathway by interacting with the 20 genes noted above to be involved in the development of melanoma.

shUBE2S depressed cell proliferation and induced apoptosis-arrested cell cycle

The total number of shUBE2S cells was shown to be significantly decreased compared with that of the shCtrl (Figure 4a and b) by a Celigo imaging assay (Nexcelom, Lawrence, MA), which showed that UBE2S knockdown depressed the proliferation of melanoma cells. The difference in cell proliferation between shUBE2S and shCtrl changed considerably at days 4 and 5 (Figure 4b). Caspase3/7 plays an important role in apoptosis and is therefore used in apoptosis evaluation. Caspase-Glo 3/7 assays showed that the Caspase3/7 activity in the shUBE2S group was significantly upregulated compared with that in the shCtrl group, which implied that shUBE2S induced the apoptosis of melanoma A375, SK-MEL-28, and MUM-2B cells on day 3 (Figure 4c and Supplementary Figures S6a and S7a). Phosphatidylserine mainly locates in the inner membrane of cells, and the translocation of phosphatidylserine to the outside membrane occurs before cell shrinkage, chromatin concentration, and DNA fragmentation. Annexin V is a calcium ion-dependent phospholipid link protein with a high affinity for phosphatidylserine that is used to detect early-cell apoptosis. Compared with the shCtrl group, the Annexin V test results showed that apoptosis was significantly increased in the shUBE2S group on day 3 (Figure 4d and Supplementary Figures S6b and S7b).

Cell cycle refers to the whole process of cell division, which contains the G0/G1 phase, the S phase, and the G2/M phase. DNA content changes according to the stage of the cycle, which can be assayed using the nuclear inserting dye...
propidium. Compared with the shCtrl group, S-phase cells decreased and G1-phase cells increased (Figure 4e and Supplementary Figures S6c and S7c) in the shUBE2S group, as shown by propidium staining. These results showed that the shUBE2S group arrested the melanoma cells in the G1/S phase.

Decreased EMT

EMT, which plays an important role in cancer metastasis, is a biological process characteristic of the cell-type transition from epithelial to mesenchymal (Gonzalez and Medici, 2014; Li and Li, 2015). During this process, adhesion molecules, such as N-cadherin, FN1, Snail, TWIST, and β-Catenin, increased, which favored the metastasis of malignant cancer cells. Using a western blot assay, N-cadherin, β-Catenin, FN1, MMP9, MYC, TWIST, Snail, VIM, and p-AKT were found to be downregulated in UBE2S knockdown melanoma cells (Figure 2), which implied that the EMT process was markedly inhibited.

Inhibition of in vivo tumor development

A tumorigenicity assay was performed in BALB/c naked mice with transfected melanoma cells by hypodermic injection. We found that the tumor volume in shUBE2S was remarkably
smaller than in shCtrl (Figure 5a). The weight of the tumor was assayed at 33 days after the mice were killed, and the tumor weight in the shUBE2S group was significantly lower than that in the shCtrl group (Figure 5e). In addition, the fluorescence area of the tumor in the shUBE2S group was smaller than that in the shCtrl group (Figure 5f). These results demonstrated that shUBE2S treatment remarkably inhibited tumor development in mice.

**DISCUSSION**

Ubiquitylation plays a vital role in the posttranscriptional modification of proteins, and its related conjugating enzymes are involved in protein degradation, signal transduction, and cancer development (Gallo et al., 2017; Ma et al., 2017; Nakayama, 2010; Wang et al., 2019). UBE2S is abnormally expressed in various cancers, such as breast cancer and metastatic colorectal cancer (Gallo et al., 2017; Wang et al., 2019), and we also found that UBE2S was overexpressed in melanoma, both cells and malignant melanoma tissue. UBE2S expression was not only associated with T staging (Supplementary Table S2) but was also negatively correlated with patient T staging (Supplementary Table S3). These data imply that UBE2S expression may be a dynamic process throughout the course of the disease, but more studies are necessary to validate these results. Preliminarily, we investigated the role of UBE2S in melanoma both in vitro and in vivo using a UBE2S knockdown platform.

**shUBE2S inhibited the cell growth, proliferation, and cell cycle of melanoma cells**

UBE2S knockdown decreased melanoma cell growth (Figure 4a and b and Supplementary Figures S6a and b and
S7a and b), which is a finding that was also reported in oral squamous cell carcinoma and pancreatic ductal adenocarcinoma (Wang et al., 2019; Yoshimura et al., 2017). Through functions analysis, differently expressed genes were found to be involved in cellular growth and proliferation processing (Supplementary Table S6). We also found that p-AKT, mTOR, and MYC were downregulated in the shUBE2S group (Figure 2). The gene for mTOR belongs to a family of phosphatidylinositol kinase-related kinases that mediate cellular responses to stresses such as DNA damage and nutrient deprivation. The mTOR pathway was involved in cell growth, proliferation, and apoptosis through gene transcription and protein synthesis. MYC is a proto-oncogene involved in cell cycle progression, apoptosis, and cellular transformation. In our study, MYC was a key factor in the gene interactive networks of different diseases and functions that interacted with other genes (Figure 3a). UBE2S knockdown may therefore suppress melanoma cell growth and proliferation.

Figure 5. Tumor growth detection in BALB/c nude mice injected with shUBE2S melanoma cells. In vivo imaging of (a) shCtrl and (b) shUBE2S BALB/c nude mice at 33 days after injection with shRNA melanoma cells. (c) Excised mouse tumors (upper roll: shCtrl; lower roll: shUBE2S). (d) The volumes of the mouse solid tumors were detected at 17, 20, 25, 28, 31, and 33 days after the injection with shRNA-treated cells. (e) Weights of solid tumors in BALB/c nude mice. (f) Fluorescence expression was detected by in vivo imaging and is displayed in a histogram. max, maximum; min, minimum; shCtrl, shRNA control cell; shRNA, short hairpin RNA; shUBE2S, UBE2S shRNA.
through the downregulation of the AKT-mTOR and MYC pathways. UBE2S is involved in Von Hippel-Lindau ubiquitin-mediated proteasomal degradation (Jung et al., 2006). Von Hippel-Lindau regulates the transcription factor hypoxia-inducible factor, which stimulates the expression of protumorgenesis and mitogenic GFs-related genes such as PGDF, VEGF, and SNAI1 (Jung et al., 2006; Rankin and Giaccia, 2016). UBE2S and HIF1α are overexpressed in several cancers such as pancreatic and mucinous colorectal breast cancers, whereas Von Hippel-Lindau is downregulated (Gallo et al., 2017; Witkiewicz et al., 2015). We also found that Snail, AKT, and MYC were downregulated in the shUBE2S group (Figure 2). UBE2S is upregulated in metastatic skin carcinoma Caki cells and human melanoma C8161 cells, and it regulates Von Hippel-Lindau degradation, promoting increased proliferation and metastasis by HIF1α and VEGF transcription (Jung et al., 2006).

Ubiquitination is involved in the cell cycle, and UBE2S plays an important role in the process of appropriate spindle formation (Ben-Eliezer et al., 2015). UBE2S elongates ubiquitin chains of anaphase-promoting complex and/or cyclosome substrates initiated by the E2 enzymes UBCH10 and UBCH5 and promotes its degradation, which leads to mitotic exit (Garnett et al., 2009). UBE2S and UBE2C regulate anaphase-promoting complex and/or cyclosome during the meiosis of mouse oocytes, and their depletions depressed the rate of initiating the first meiotic cytokinesis by 50% (Ben-Eliezer et al., 2015). UBE2S knockdown arrested the cell cycle in the G2/M phase in oral squamous cell carcinoma cell lines through the promotion of P21 degradation (Yoshimura et al., 2017). In our study, the cell cycle was arrested in the G1/S phase in the shUBE2S group (Figure 4e). Cycle-dependent kinase inhibitor p27Kip1 negatively regulates G1 to S phase (Frescas and Pagano, 2008; Morgan, 1995; Sherr and Roberts, 1999). p27Kip1 is downregulated in protein level in the late G phase by ubiquitination-mediated degradation (Pagano et al., 1995). A common mutation of BRAFV600E in melanoma inhibits the expression of p27Kip1 (Bhatt et al., 2007). To summarize, UBE2S regulates the melanoma cell cycle through the downregulation of cycle-dependent kinase with p27Kip1. Not limited to G1/S phase arrest, we also found that the UBE2S knockdown induced the apoptosis of melanoma cells, which may be regulated through the AKT-mTOR pathway and MYC (Figures 2 and 3a). Drug resistance to BRAF/MEK inhibitors and relapses are associated with the downstream activation of MAPK pathway and activation of parallel signaling pathways (phosphoinositol-3-kinase/AKT) (Flaherty et al., 2012). In our study, the downregulation of the NRAS gene (Supplementary Tables S4) and ERK5 signaling in the shUBE2S group was observed, which implied that UBE2S may be a potential target for BRAF inhibitor-resistant patient. It is worthwhile to study the application of UBE2S small interfering RNA monotherapy or as a combined therapy with BRAF inhibitor in patients with melanoma.

**Influenced immune activity**

E3 ligase awakens the innate immune system to stop cancer metastasis (Paolino et al., 2014). E3 ligase recruits both E2-ubiquitin complex and its substrate, which is involved in NK cell anergy induction by promoting mono-ubiquitination of CARMA1. NK cells recognize and kill melanoma cells after the antitumor reactivity of NK cells is activated by the inhibition of E3 ligase (Gallo et al., 2017). However, the function of UBE2S in immune activity is not clear. We found that 10 upregulated genes in the shUBE2S group were involved in IFN signaling (Table 1). A total of 18 differentially expressed genes were involved in inflammatory demyelinating disease (Table 1). Using regulator effect analysis, 16 differently expressed genes were involved in the accumulation of granulocytes and the accumulation of phagocytes functions (Supplementary Table S6). We found that the LXR/RXR signaling pathway was activated, and nine genes were involved in this signaling (Table 1). The LXR/RXR signaling pathway is not only involved in inflammatory responses and cholesterol and glucose metabolism (A-González and Castrillo, 2011) but also in a variety of malignancies (Lin and Gustafsson, 2015). It is downregulated in prostate cancer and colon adenocarcinoma (Davali et al., 2017; Tang et al., 2014). LXR agonists inhibited the proliferation of prostate cancer cells, decreased S-phase cells, and increased the expression of p27Kip1 (Fukuchi et al., 2004). The LXR/RXR signaling pathway may be a new target for controlling tumor cell proliferation. Our results imply that UBE2S may regulate melanoma tumor development through immune activity, including interferon signaling, LXR/RXR activation, and accumulation of immune cells, by regulating FOS, IFI35, TNFRSF11B, IFIT1, and 16 other molecules (Figure 3b).

NF-κB pathway was inhibited in the shUBE2S group (Table 1), and proteins expression of NF-κB-p65 and p-AKT were downregulated in the shUBE2S group (Figure 2). The AKT pathway regulates the expression of Notch1 by NF-κB activation. Notch1 activity increases the melanoma growth and metastatic rates (Balint et al., 2005), and the Notch signal is regulated by ubiquitination (Gupta-Rossi et al., 2001). NF-κB regulates growth and inflammation and inhibits apoptosis (Perkins, 2007), but NF-κB is inhibited by iκB. NF-κB is released when iκB is degraded in a ubiquitination-dependent manner by phosphorylation. Lys63-linked ubiquitin chains are built by ubiquitin-conjugating enzyme E2N and its cofactor UBE2V1, which are essential in the NF-κB inflammatory activation process (Chen and Chen, 2013). Decreased UBE2S and ubiquitin-conjugating enzyme E2N may restrain the melanoma growth through the restraint of the NF-κB inflammatory pathway. It was reported that the activation of the AKT-mTOR pathway promoted immune escape by driving the expression of PD-L1 in a mouse model of lung cancer (Lastwika et al., 2016). A PD-1 antibody combined with an mTOR inhibitor downregulated tumor growth, increased the number of tumor-infiltrating T cells, and suppressed the production of regulatory T cells (Lastwika et al., 2016).

Downregulation of the AKT-mTOR pathway in the shUBE2S group (Figure 2) may provide synergistic therapeutic effects with immunotherapy, although more research on the combination of UBE2S small interfering RNA with an AKT-mTOR inhibitor and an anti-PD-1 inhibitor therapies in melanoma is needed.
EMT and DNA repair are also involved in tumor suppression

EMT decreases cell adhesion and polarity and promotes cell detachment in tumor metastasis (Cheung and Ewald, 2016). Downregulated EMT-related proteins in the shUBE2S group, such as N-cadherin, β-Catenin, VIM, CDH2, FN1, MMP9, MYC, TWIST, and Snail, showed that EMT was inhibited (Figure 2) during melanoma development. UBE2S knockdown may suppress melanoma cell development, and tumor growth was inhibited through EMT inhibition in the shUBE2S-transfected nude mice, as shown in the tumorigenicity assay (Figures 2 and 5).

Ubiquitin modification plays an important role in the repair of DNA damage, and UBE2S is responsible for the assembly of Lys11-linkage conjugation on damaged chromatin at the site of DNA damage (Paul and Wang, 2017). We found that 31 differentially expressed genes were involved in RNA posttranscriptional modification, protein synthesis, DNA replication, recombination, and repair. Among these genes, FOS, IL36G, NRAS, PTGS2, and TNRFSF11B were involved in PPAR signaling (Table 1). Overexpressed UBE2S may regulate cell growth through DNA repair pathways in melanoma development. AKT1-phosphorylated UBE2S at the site of Thr 152 resulted in the accumulation of UBE2S, which was associated with the components of the nonhomologous end-joining complex and involved in the nonhomologous end-joining-mediated DNA repair process in the double-stranded break sites in response to etoposide treatment. Nonhomologous end-joining-mediated double-stranded break repair was inhibited, and glioblastoma cells were more sensitive to chemotherapy with UBE2S knockdown (Hu et al., 2017).

In summary, UBE2S knockdown arrested the cell cycle in the G1/S phase, inhibited melanoma cell proliferation, induced apoptosis, and suppressed tumorigenicity through EMT suppression, DNA damage repair processes, and the inhibition of the AKT-mTOR pathway, ERK signaling, NF-κB signaling, and LXR/RXR activation (Supplementary Figure S8). These findings imply that UBE2S is an important molecular target for melanoma therapy because it is involved in the AKT-mTOR pathway and LXR/RXR activation, which may also be potential targets for tumor inhibition. The role of UBE2S in melanoma advances our understanding of the molecular mechanisms of melanoma development and provides a few new potential targets for melanoma therapy.

MATERIALS AND METHODS

Permission for these studies was obtained from the medical ethics committee (The Third People's Hospital of Hangzhou, China). All animal experiments complied with the National Institutes of Health guide for the care and use of laboratory animals.

Detailed experimental materials and methods are described in the Supplementary Materials online; therefore, we briefly present the general methods here. Melanoma tissue arrays containing malignant melanoma and normal skin tissue were assayed using immunohistochemistry. The associations between UBE2S expression and T stage, clinical stage, and cancer types were also analyzed. We designed an interference sequence for UBE2S (accession: NM_014501) and constructed a vector, GV115. Then, the melanoma cell lines of A375, SK-MEL-28, and MUM-2B were transfected with the lentivirus. Cell growth was assayed using Celigo. Apoptosis was detected using Caspase-Glo 3/7 and Annexin V assays. Cell cycle stages were detected using propidium staining under flow cytometry. Gene expression profiling was investigated using Human GeneChip primemixion (Affymetrix, Santa Clara, CA), and the involved pathways were analyzed using IPA. The roles of shUBE2S on tumor development in vivo were investigated using BALB/c nude female mice. The associations between UBE2S expression and T stage, clinical stage, and cancer types were also analyzed.

Data availability statement

mRNA expression data are publicly available and have been deposited in the Gene Expression Omnibus, accession no. GSE115922.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: PW, YL; Data Curation: PW, YL, YM, ZL, WY; Formal Analysis: YL, MZ, JW; Funding Acquisition: PW, YL, YX; Investigation: PW, YL, YM, ZL, WX, WY; Methodology: XZ, ZL, WY, Project Administration: PW, YL, YX, AX; Resources: PW, YL, YX, AX; Visualization: YM, JW; Writing - Original Draft Preparation: PW, YL, XZ, MZ; Writing - Review and Editing: PW, YL, YM, XZ, ZL, WX, MZ, JW, YX, AX

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.05.113.

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