



# EMG1 interacts with NOP14 to regulate the growth, migration, and invasion of melanoma cells via the Wnt/ $\beta$ -catenin pathway

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**Background:** The role of EMG1 in the progression of malignant melanoma remains unclear.

**Methods:** Expression levels of EMG1 in melanoma tissues from GEO and TCGA databases was analyzed. The role of EMG1 was determined by overexpression on cell growth, apoptosis, migration and invasion. Glutathione S-transferase (GST) pulldown and co-immunoprecipitation (CoIP) assays were applied to reveal the relationship of EMG1 and nucleolar complex protein 14 (NOP14).

**Results:** The expression level of EMG1 was downregulated in melanoma tissues in GSE7553 dataset and further decreased in tissues from metastasis patients from both GEE7553 and TCGA cohorts. Overexpression of EMG1 suppressed proliferation, promoted apoptosis, and inhibited migration and invasion in melanoma cells. EMG1 interacted with NOP14 and functioned together to regulate the growth, apoptosis, migration and invasion of cultured melanoma cells. Furthermore, simultaneous overexpression of EMG1 and NOP14 decreased the levels of WNT3a,  $\beta$ -catenin, phosphorylated-GSK-3 $\beta$ , and c-Myc.

**Conclusions:** EMG1 and NOP14 inhibit melanoma cell proliferation, migration, and invasion by regulating the Wnt/ $\beta$ -catenin signaling pathway.

**Keywords:** Melanoma; EMG1; nucleolar complex protein 14 (NOP14); Wnt/ $\beta$ -catenin; cancer progression

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## Introduction

Malignant melanoma, which originates from melanocytes located in the skin or mucous membranes, is an invasive cancer with a high fatality rate (1). The incidence of malignant melanoma has increased steadily in recent years, giving it one of the highest mortality rates among human cancers (1,2). Age and family history, exposure to ultraviolet radiation, fair skin, and dysplastic nevi syndrome are the known risk factors for malignant melanoma progression (3), and the typical characteristics of melanoma are early metastasis and uncontrolled growth (4). Surgery, radiation therapy, and chemotherapy, the current therapeutic

interventions for metastatic melanoma, are insufficient to achieve durable responses (5). Therefore, combinations of treatment modalities, including immunotherapies and molecularly targeted therapies, must be explored for the treatment of advanced melanoma (6,7). Although some treatments have shown clinical benefit, understanding the mechanism of melanoma progression will reveal additional targets (8). Hence, identifying new biomarkers and exploring the mechanisms involved in melanoma progression are critical for the development of novel melanoma therapies.

Nucleolar complex protein 14 (NOP14) is a nucleolar stress response protein responsible for 18S rRNA maturation and 40S ribosome production (9). Because ribosomes play

an important role in DNA repair and in the regulation of cell proliferation, disorders of ribosome function can lead to tumor formation (10). Mounting evidence suggests that NOP14 is involved in the development of cancer. For instance, overexpression of NOP14 promoted the proliferation and metastasis of pancreatic cancer lines *in vitro* and *in vivo* (11). By inhibiting the nuclear receptor-interacting protein 1 (NRIP1)/Wnt/ $\beta$ -catenin pathway, NOP14 suppressed oncogenesis and metastasis in a breast cancer model (12). Warda *et al.* identified a nucleolar subcomplex containing the pre-ribosomal factors NOP14, nucleolar complex protein 4 homolog (NOC4L), UTP14A, and EMG1, which was required for its recruitment to nucleoli. The D86G mutation of EMG1, which is associated with Bowen-Conradi syndrome, resulted in its decreased nucleolar localization, proteasome-dependent degradation, and accumulation in nuclear foci. In addition to its role in nuclear import, binding of the importin (Imp)  $\beta$ /7 heterodimer to EMG1 can prevent its non-specific aggregation with RNAs *in vitro*, suggesting that importins may act as chaperones by binding to RNA methyltransferases (13). Furthermore, EMG1 is involved in ribosome biogenesis. The striking phenotype in lymphoblasts compared with fibroblasts suggests a greater need for EMG1 in rapidly dividing cells (14). However, the role of NOP14 and EMG1 in melanoma development remains largely exclusive.

The Wnt/ $\beta$ -catenin axis orchestrates numerous biological processes, including cell proliferation, differentiation, tissue regeneration, organogenesis, and tumorigenesis (15-19). Targeting the versatile Wnt/ $\beta$ -catenin pathway is considered a promising potential strategy for cancer therapy (20,21).  $\beta$ -catenin is a key transducer of Wnt signaling (22,23). A complex consisting of adenomatous polyposis coli (APC), casein kinase 1 $\alpha$  (CK1 $\alpha$ ), glycogen synthase kinase 3 $\alpha$ / $\beta$  (GSK-3 $\alpha$ / $\beta$ ), and AXIN1 tightly controls  $\beta$ -catenin by phosphorylation-mediated proteolysis (22,24-27). Some studies have suggested that during melanomagenesis, the  $\beta$ -catenin transcription factor is frequently activated via non-mutational alterations such as reduced expression of CK1 $\alpha$ , leading to  $\beta$ -catenin protein stabilization (28,29). Some studies indicated that  $\beta$ -catenin inhibits melanoma cell invasiveness, and the loss of  $\beta$ -catenin predicts poor survival in melanoma patients (30-33). Other studies showed both *in vitro* and *in vivo* that increased abundance or stabilization of  $\beta$ -catenin results in increased melanoma metastasis (34-38).

In this study, we found that EMG1 interacts with NOP14 to regulate the growth, migration, and invasion of melanoma cells by regulating Wnt/ $\beta$ -catenin signaling.

These findings may suggest EMG1 and NOP14 as novel targets for the treatment of melanoma.

## Methods

### *Cell culture and treatment*

The human melanoma cell lines A375 and SK-MEL-1 were from the Cell Bank of the Chinese Academy of Sciences (Shanghai). As previously reported (39), all cell lines were cultured in DMEM (Thermo Fisher Scientific, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 100 U/mL each of penicillin and streptomycin (Gibco/Thermo Fisher Scientific, USA) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Full-length human cDNAs were amplified by PCR to construct EMG1- or NOP14-encoding plasmids using the pcDNA3.1 vector as the backbone (Realgene, China). Cells (1 $\times$ 10<sup>5</sup> cells/well) were plated in 24-well plates and transfected with NOP14 or EMG1 overexpression plasmids using FuGENE HD transfection reagent (Roche Applied Science, USA). The cells were harvested for analysis after 48 h of transfection.

### *Migration and invasion assays*

Cells (2 $\times$ 10<sup>5</sup>) were plated in the upper wells of Transwell inserts (Corning, USA) with (for invasion experiments) or without (for migration experiments) 10 mg/mL Matrigel (BD Biosciences, USA). The lower wells of the Transwell chambers were filled with culture medium containing 10% FBS. Non-migrating cells on the upper side of the filter were removed by wiping with a cotton swab after 48 h of incubation at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. The membranes were fixed with 70% cold ethanol, and cells were stained with 0.1% crystal violet. Cell counting was performed under 200 $\times$  magnification using a light microscope (Olympus, Japan). The experiment was repeated three times.

### *Western blotting*

Cells were lysed in ice-cold mammalian radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China) containing a protease inhibitor cocktail (Invitrogen/Thermo Fisher Scientific, USA). Protein quantification was performed using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). SDS-PAGE was used to separate equal amounts of protein, which were transferred onto polyvinylidene fluoride (PVDF)

membranes (Thermo Fisher Scientific) and then incubated with 10% non-fat milk overnight at 4 °C. Membranes were washed three times with phosphate-buffered saline (PBS) containing Tween 20 (PBST) and then incubated for 1 h at room temperature with the following primary antibodies: EMG1 (1:1,000), NOP14 (1:500), WNT3a (1:800),  $\beta$ -catenin (1:1,000), GSK-3 $\beta$  (1:500), and GAPDH (1:2,000). The antibodies were all purchased from Abcam (USA). After washing three times with PBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG H + L secondary antibodies (Southern Biotech, USA) at a 1:5,000 dilution. An enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific) was used to visualize protein bands.

### **Immunoprecipitation**

Protein from  $1 \times 10^7$  A375 or SK-MEL-1 cells was extracted in 1 mL RIPA buffer containing 0.5% NP-40, 1.0% Triton X-100, and protease inhibitors, and was collected by centrifugation at 14,000  $\times g$  for 15 min at 4 °C. The extract was precleared by incubation with 5  $\mu g$  of normal goat IgG. The interacting proteins were co-immunoprecipitated (CoIPed) using EMG1 and NOP14 antibodies (Abcam), and SDS-PAGE was performed for western blotting.

### **GST pulldown assays**

NOP14 and EMG1 expression plasmids were cloned into the pGEX-2T prokaryotic expression vector downstream of the glutathione S-transferase (GST) sequence. Then, plasmids were used to transform the *E. coli* BL21 strain, and protein expression was induced using 1 mM isopropylthiogalactoside (IPTG) for 1 h at 37 °C. Bacteria were then pelleted and resuspended in PBS supplemented with complete protease inhibitors (Roche Applied Science), and lysates were collected by centrifugation (10,000  $\times g$ , 15 min, 4 °C). Purified GST-NOP14 and -EMG1 proteins were incubated with beads coupled with approximately 5  $\mu g$  glutathione-sepharose. Proteins bound to the beads were separated by 10% SDS-PAGE, and gels were stained with Coomassie brilliant blue R-250. Bands representing putative NOP14- and EMG1-binding proteins were recovered, digested by trypsinization, and analyzed by western blotting.

### **Flow cytometry**

Apoptosis was assessed using an annexin V and propidium

iodide (PI) dual staining kit (Biolegend, USA) according to the manufacturer's instructions. Briefly, cells ( $5 \times 10^3$ ) were resuspended in 500 mL binding buffer. Then, 5  $\mu L$  each of annexin V-FITC and PI was added to the cell suspension. After 15 min of incubation at room temperature, the cells were analyzed by flow cytometry. The experiment was repeated in triplicate.

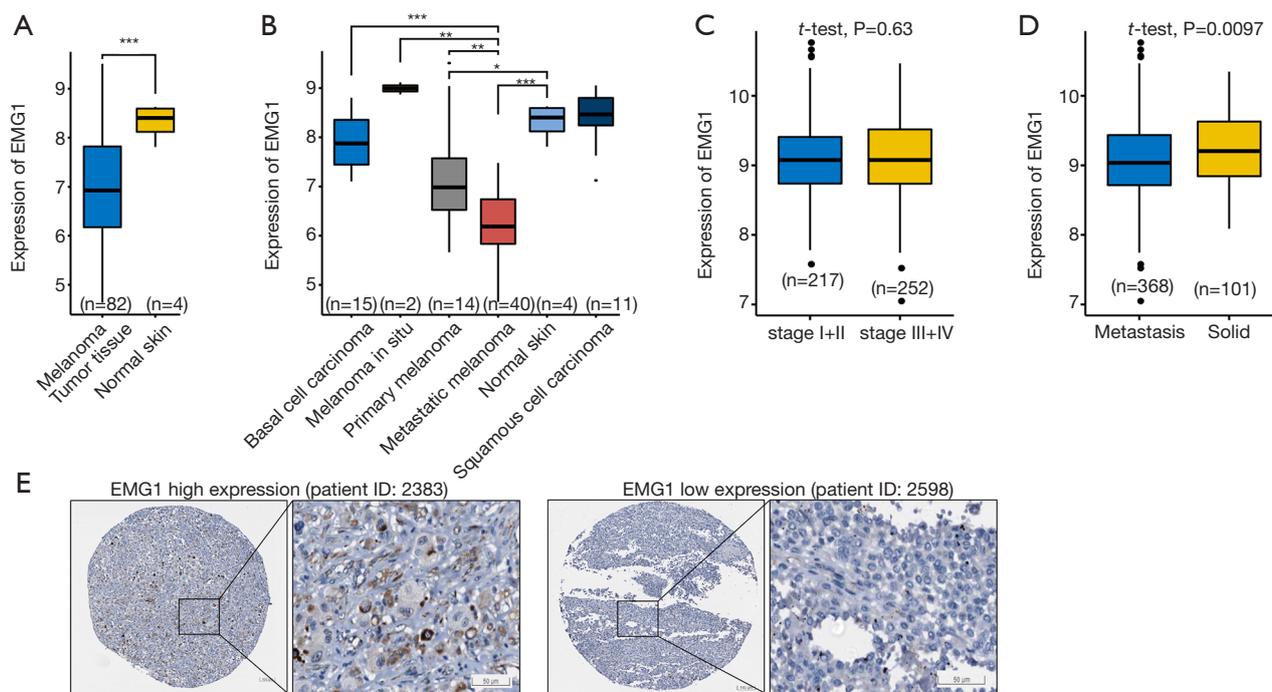
### **Statistical analysis**

SPSS 21.0 software (IBM, USA) was used for all statistical analysis. Data represent the mean  $\pm$  standard deviation (SD). Student's *t*-tests were used for comparisons of two groups, and one-way analysis of variance (ANOVA) was used for multiple group comparisons. The correlations between EMG1 protein levels and clinicopathological characteristics of melanoma patients were analyzed by chi-squared tests.  $P < 0.05$  was considered statistically significant.

## **Results**

### **EMG1 expression is associated with melanoma**

EMG1 expression in the Riker melanoma dataset was analyzed using the public database Oncomine (40). EMG1 mRNA expression was significantly lower in melanoma than in normal skin (*Figure 1A*). Further, we downloaded the cohort (GSE7553) and analyzed the subtype groups. The results showed that EMG1 was downregulated in primary melanoma comparing to normal skin, and the level of EMG1 in metastatic melanoma was further decreased, comparing to other melanoma types (*Figure 1B*). Moreover, we explored TCGA database to analysis the expression levels of EMG1 and found that the expression level of EMG1 showed no significant difference between the groups of stage I + II and III + IV patients (*Figure 1C*). However, the expression level was significantly downregulated in patient group with metastasis (*Figure 1D*). In the Human Protein Atlas (HPA) database (41), a patient (ID: 2598) with melanoma progression had low EMG1 levels, while a non-progressing patient (ID: 2383) had high EMG1 progression (*Figure 1E*). Using online tools from PROGgene (42), oncoLnc (43), HPA and Gepia (44), the overall survival rate was analyzed between patients with different expression levels of EMG1, however, the results all showed no significance (data not shown). Taken together, these mining data reveal that EMG1 is downregulated during melanoma development.



**Figure 1** EMG1 expression is associated with melanoma development. EMG1 expression in the Riker melanoma dataset from the published tumor-related gene database Oncomine (A) and in subtype melanoma tissues (B). (C,D) EMG1 expression levels in different groups of patients from TCGA database was analyzed. (E) EMG1 immunohistochemistry results from Human Protein Atlas. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

### ***EMG1 overexpression inhibits melanoma cell proliferation, migration, and invasion***

To evaluate the functional role of EMG1 in melanoma, EMG1 was overexpressed in two melanoma cell lines, A375 and SK-MEL-1. The upregulation of EMG1 was confirmed by western blotting (Figure 2A,B). Overexpression of EMG1 significantly inhibited the growth of the two melanoma cell lines (Figure 2C,D). Overexpression of EMG1 also significantly increased apoptosis (Figure 2E,F), while migration (Figure 2G,H) and invasion (Figure 2I,J) were both inhibited by EMG1 overexpression. Taken together, these data indicate that EMG1 inhibits proliferation, migration, and invasion in melanoma cells.

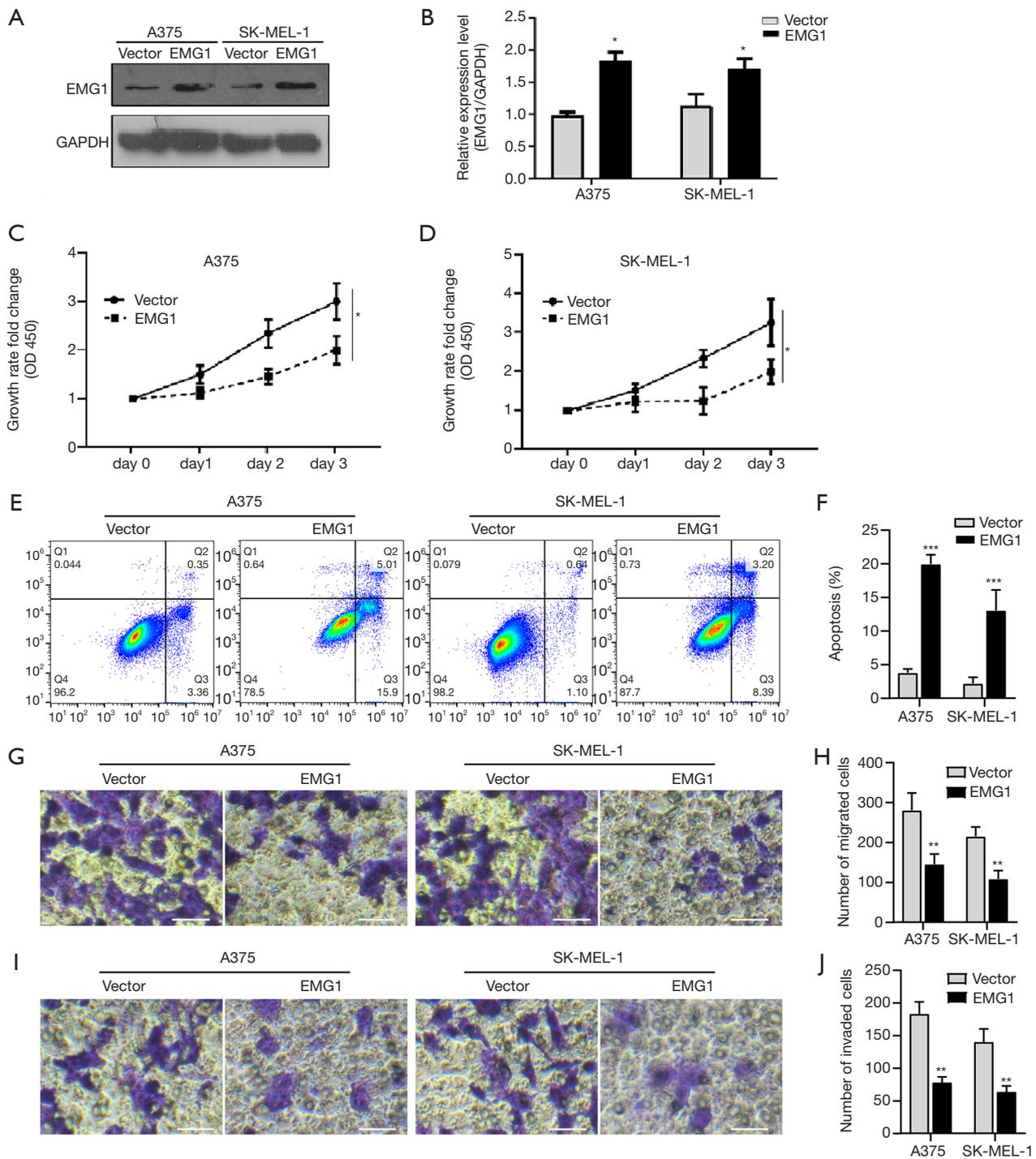
### ***EMG1 associates with NOP14***

A potential interaction between EMG1 and NOP14 has been reported; however, this interaction has not been evaluated in melanoma. EMG1-interacting proteins were therefore evaluated by GST pulldown and CoIP assays in melanoma cells. For GST pulldown assays, two GST fusion

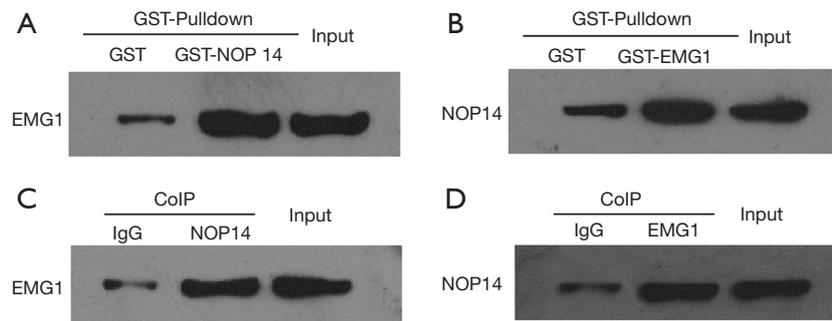
plasmids, GST-EMG1 and GST-NOP14, were constructed. EMG1 was pulled down with GST-NOP14 (Figure 3A), and NOP14 was pulled down with GST-EMG1 (Figure 3B). Furthermore, EMG1 CoIPed with an NOP14 antibody (Figure 3C), and NOP14 CoIPed with an EMG1 antibody (Figure 3D). Taken together, these data suggest that EMG1 interacts with NOP14 in melanoma cells.

### ***EMG1 and NOP14 function together to suppress the progression of melanoma***

After determining that EMG1 interacts with NOP14 in melanoma cells, we asked whether EMG1 and NOP14 act together to control the progression of melanoma. A375 and SK-MEL-1 melanoma cells overexpressing EMG1, NOP14, or the combination were generated using previously verified overexpression plasmids (39). Cell proliferation, apoptosis, migration, and invasion were observed. As shown in Figure 4A and B, compared with the control group, A375 cells overexpressing NOP14 or EMG1 showed significantly decreased proliferation, while



**Figure 2** EMG1 inhibits progression in melanoma. (A) EMG1 is overexpressed in A375 and SK-MEL-1 cells. (B) Quantification of the western blotting results in (A). (C) Growth rate of EMG1-overexpressing A375 cells. (D) Growth rate of EMG1-overexpressing SK-MEL-1 cells. (E) Measurement of apoptosis in the two cell lines after overexpression of EMG1. (F) Quantification of the flow cytometry results. (G) Migration of the two cell lines. (H) Quantification of the migration assay results. (I) Invasion of the two cell lines. (J) Quantification of the invasion assay results. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 versus Vector. Scale bar: 20  $\mu$ m.



**Figure 3** EMG1 associates with NOP14. (A) GST-NOP14 pulldown for EMG1. Protein levels of EMG1 were detected by western blotting. (B) GST-EMG1 pulldown for NOP14. Protein levels of NOP14 were detected by western blotting. (C) CoIP of EMG1 with a NOP14 antibody. Protein levels of EMG1 were detected by western blotting. (D) CoIP of NOP14 with an EMG1 antibody. Protein levels of NOP14 were detected by western blotting. NOP14, nucleolar complex protein 14.

cells overexpressing both NOP14 and EMG1 showed a further decrease in proliferation (*Figure 4A*). Similar results were obtained with the SK-MEL-1 cells (*Figure 4B*). The apoptosis rate in both cell lines was also significantly increased by overexpression of EMG1 or NOP14, and the strongest effects were observed when both proteins were overexpressed together (*Figure 4C,D*). Similar trends were observed with respect to cell migration (*Figure 4E,F*) and invasion (*Figure 4G,H*). These data suggest that EMG1 and NOP14 act together to inhibit cell proliferation, migration, and invasion in melanoma cells.

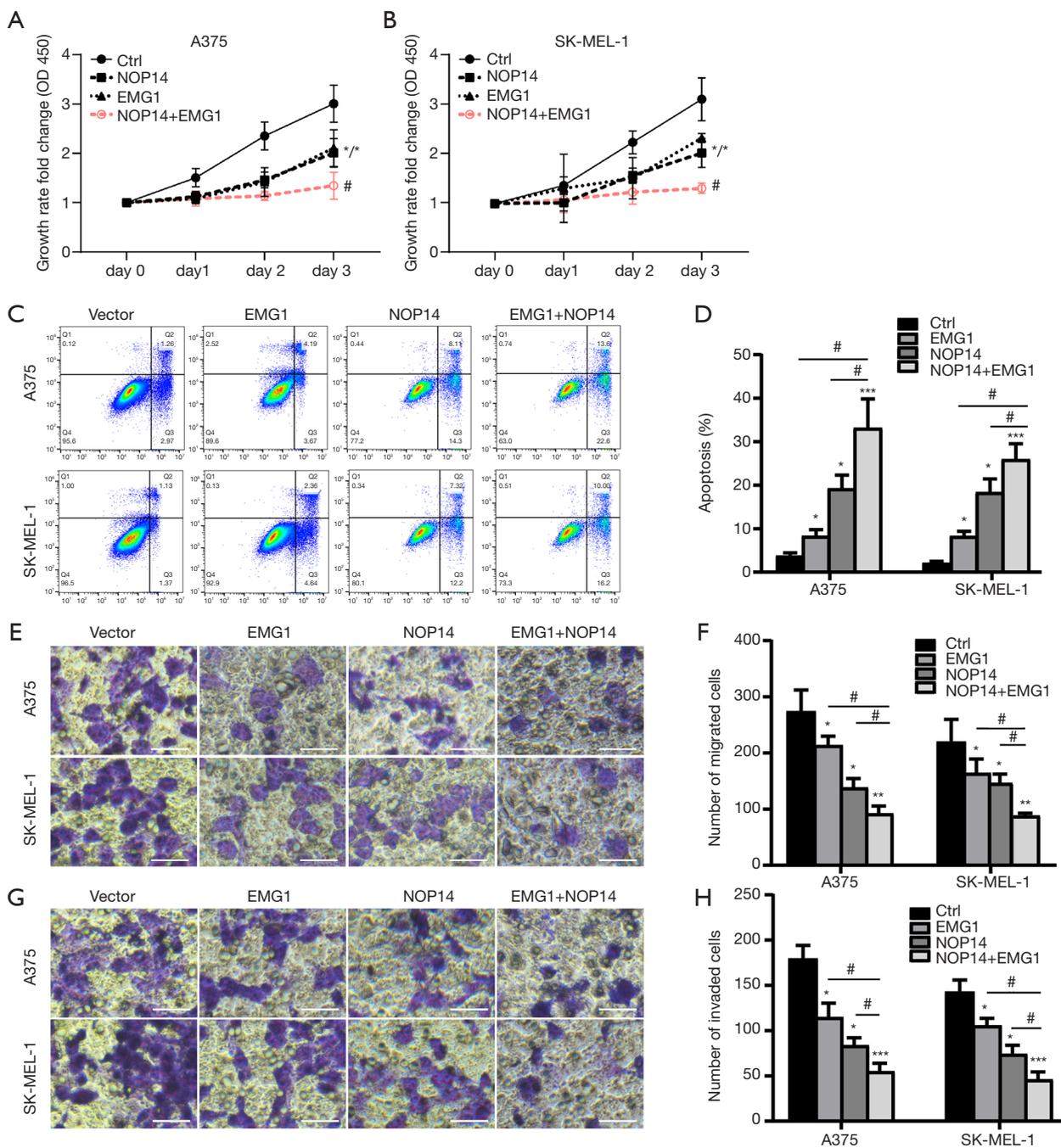
#### *EMG1 and NOP14 modulate Wnt/ $\beta$ -catenin signaling in melanoma cells*

After validating the functional role of EMG1 and NOP14 in melanoma, we evaluated the detailed mechanism through which EMG1 and NOP14 alter melanoma development. It has been reported that the Wnt/ $\beta$ -catenin signaling pathway plays an important role in cancer progression, including in melanoma. In our previous studies, we evaluated changes in the mRNA and protein levels of genes encoding various components of the Wnt/ $\beta$ -catenin pathway in response to overexpression of NOP14 in melanoma cell lines (39). We asked whether EMG1 and NOP14 would show increased effects on this same pathway. The levels of WNT3a,  $\beta$ -catenin, and phosphorylated GSK3 $\beta$  (p-GSK-3) were evaluated by western blotting. In addition, the level of c-Myc, a transcriptional target of the Wnt/ $\beta$ -catenin pathway that is thought to modulate the expression of genes involved in melanoma development, was also evaluated (45). As shown in *Figure 5*, overexpression of NOP14, alone or

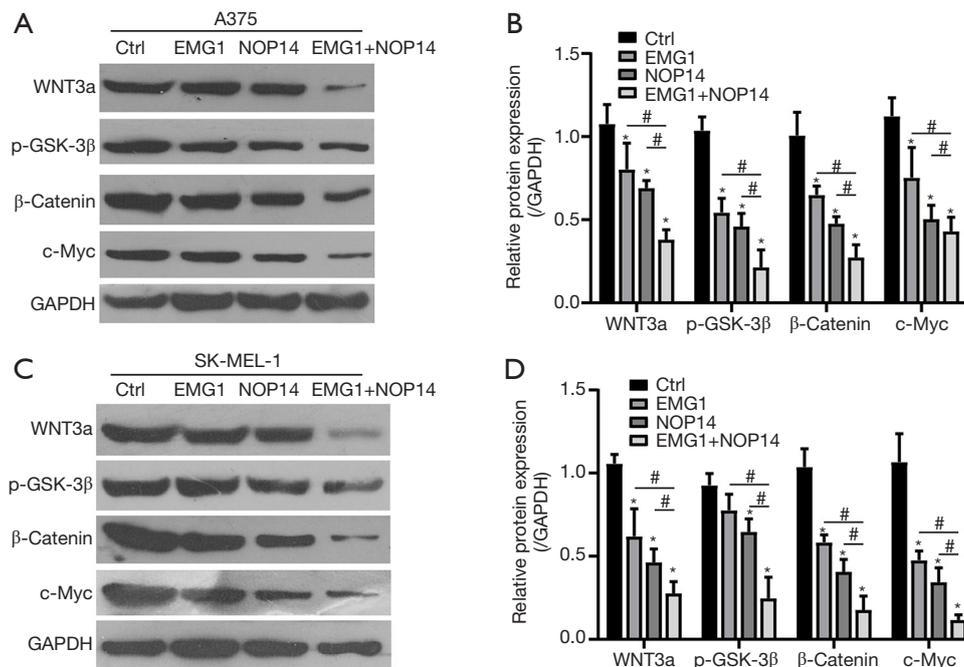
together with EMG1, in the A375 cell line significantly suppressed the expression of WNT3a, p-GSK-3 $\beta$ ,  $\beta$ -catenin, and c-Myc. In addition, the levels of these proteins decreased more significantly in cells in which both NOP14 and EMG1 were overexpressed (*Figure 5A,B*). Similar results were observed in the SK-MEL-1 cell line (*Figure 5C,D*). These results suggest that EMG1 and NOP14 act together to regulate melanoma development by modulating the Wnt/ $\beta$ -catenin signaling pathway.

#### **Conclusions**

In recent years, there has been accumulating evidence that NOP14 is involved in tumor progression, cell proliferation, migration, invasion, and apoptosis (46-49). In a previous study, we found that in malignant melanoma, NOP14 was downregulated compared with the level of expression in melanocytes (50). In addition, we identified a significant correlation between NOP14 expression and melanoma thickness and lymph node metastasis. NOP14 is a nuclear protein involved in the processing of pre-18S rRNA and in small ribosomal subunit assembly (9). Its function is highly conserved in eukaryotic organisms (51). NOP14 is essential for 40 s ribosome maturation. Heat stress can cause a sharp decrease in the expression of NOP14 mRNA, thereby reducing the level of 40 s ribosomal subunits (51). Nop14p, the yeast homolog of human NOP14 (9,52), is a small subunit (SSU) biogenesis factor that is a component of the pre-90S particle (53). Loss of Nop14p decreased the levels of 20S and 27SA2 pre-rRNA, and increased the production of 35S and 23S pre-rRNA (9,52). NOP14 is known to regulate the expression of EMG1 [also known as Nep1 (54)].



**Figure 4** EMG1 and NOP14 act together to block the progression of melanoma. (A) Growth rate of A375 cells overexpressing NOP14, EMG1, and NOP14 + EMG1. (B) Growth rate of SK-MEL-1 cells overexpressing NOP14, EMG1, and NOP14 + EMG1. (C) Apoptosis in the two cell lines after overexpression of NOP14, EMG1, and NOP14 + EMG1. (D) Quantification of the flow cytometry results. (E) Migration of the two cell lines. (F) Quantification of the migration assay results. (G) Invasion of the two cell lines. (H) Quantification of the invasion assay results. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 versus the control group; #P<0.05 versus the NOP14 + EMG1 group. NOP14, nucleolar complex protein 14. Scale bar: 20  $\mu$ m.



**Figure 5** EMG1 and NOP14 overexpression suppresses the Wnt/ $\beta$ -catenin pathway in melanoma cells. (A) Western blotting for WNT3a, p-GSK-3 $\beta$ ,  $\beta$ -catenin, and c-Myc in A375 cells. (B) Quantification of the western blotting results. (C) Western blotting for WNT3a, p-GSK-3 $\beta$ ,  $\beta$ -catenin, and c-Myc in SK-MEL-1 cells. (D) Quantification of the western blotting results. \* $P < 0.05$  versus the control group; # $P < 0.05$  versus the NOP14 + EMG1 group. NOP14, nucleolar complex protein 14.

EMG1 is involved in processing rRNA precursor molecules into 18s RNA, and EMG1 interacts with NOP14 to promote the maturation of 18sRNA and the production of 40sRNA (51). The yeast ortholog of EMG1 is required for the biogenesis of small ribosomal subunits, which are involved in the hyper-modification of uridine 1191 of 18S rRNA (51,55,56). Consistent with this, EMG1 was recently found to bind to this site in the 90S pre-ribosomes of *Chaetomium thermophilum* by cryo-electron microscopy (cryo-EM) (57,58).

After further evaluation of the functions of EMG1 in the A375 and SK-MEL-1 melanoma cell lines, we hypothesized that EMG1 overexpression suppressed melanoma cell proliferation. EMG1 overexpression also increased apoptosis in both melanoma cell lines. In Transwell assays, EMG1 overexpression decreased the migratory capacity and invasiveness of A375 and SK-MEL-1 cells. These data suggest that EMG1 plays an important role in melanoma formation and development.

The Wnt proteins are widely expressed in invertebrates and vertebrates, and are highly conserved during species evolution. Wnt signaling plays a crucial role in the early

development of animal embryos, organ formation, tissue regeneration, and other physiological processes (59). It is now widely accepted that inappropriate positioning of  $\beta$ -catenin in the nucleus is a key carcinogenic process.  $\beta$ -catenin is susceptible to transfer to the nucleus, and systems that regulate nuclear  $\beta$ -catenin entry are multifactorial and highly dependent on the environment (60). Recent studies have determined that melanoma cells develop an efficient new mechanism to activate the  $\beta$ -catenin signaling pathway by suppression of CK1 $\alpha$  expression, defining CK1 $\alpha$  as a novel tumor suppressor in melanoma (28,61). The activity of  $\beta$ -catenin is mainly determined by regulation of its proteolysis by the  $\beta$ -catenin destruction complex, which includes CK1 $\alpha$ , GSK-3, APC, and AXIN1 (26,62). Constitutive activation of signal transducer and activator of transcription 3 (STAT3) promotes survival in a wide spectrum of human cancers, including malignant melanoma (63). The study of these mechanisms may have important therapeutic implications. The activity of the Wnt/ $\beta$ -catenin signaling pathway depends primarily on the activity of GSK-3, which controls  $\beta$ -catenin stability/degradation (64). GSK-3-dependent phosphorylation of  $\beta$ -catenin restricts its nuclear translocation by inducing

proteasome-dependent proteolysis (65). In addition, it has been reported that the Wnt/ $\beta$ -catenin signaling pathway also contributes to metastasis in melanoma (66). Here, we show that co-expression of EMG1 and NOP14 inhibits the Wnt/ $\beta$ -catenin pathway to suppress melanoma development. Targeting EMG1 and NOP14 may therefore be a potential strategy for the treatment of melanoma patients with abnormally activated Wnt/ $\beta$ -catenin signaling.

In conclusion, we report that EMG1 is downregulated in malignant melanoma. EMG1 and NOP14 overexpression suppresses melanoma cell proliferation, promotes apoptosis, and inhibits cell migration and invasion. Additionally, overexpression of EMG1 and NOP14 decreases the level of WNT3a, p-GSK-3 $\beta$ ,  $\beta$ -catenin, and c-Myc. Thus, EMG1 interacts with NOP14 to reduce cell proliferation and metastasis by regulating the Wnt/ $\beta$ -catenin signaling pathway in melanoma. These data may provide new support for the development of therapeutic agents targeting these molecules in melanoma.

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### Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.03.79>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work, and will ensure that all questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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