Nectin-4 promotes osteosarcoma progression and metastasis through activating PI3K/AKT/NF-κB signaling by down-regulation of miR-520c-3p

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Research Article

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Abstract

Purpose

Nectin-4 was specifically up-regulated in various tumors, exerted crucial effects on tumor occurrence and development. Nevertheless, the role and molecular mechanism of Nectin-4 in osteosarcoma (OS) were rarely studied.

Methods

The expression of Nectin-4 and its relationship with clinical characteristics of OS were investigated using OS clinical tissues, tissue microarrays, TCGA and GEO databases. Moreover, the effect of Nectin-4 on cell proliferation, migration, and invasion abilities were detected by *in vitro* functional assays, respectively. The RT-qPCR, Western blotting and luciferase reporter assays were performed to explore molecular mechanism which Nectin-4 mediated the expression of miR-520c-3p, thereby modulating PI3K/AKT/NF-κB signaling. *In vivo* mice models were used to validate the functional roles of Nectin-4 and miR-520c-3p.

Results

Nectin-4 displayed a higher expression in OS tissues compared with normal tissues. We also found that Nectin-4 overexpression was positively associated with tumor stage and metastasis in OS patients. Besides, the miR-520c-3p was negatively correlated with Nectin-4, and its low expression is closely related with tumor metastasis in OS patients. Functionally, Nectin-4 enhanced cells proliferation and cell mobility *in vitro*. Moreover, mice models with subcutaneous xenograft and lung metastasis confirmed that knockdown of Nectin-4 could suppress the tumorigenesis and tumor metastasis *in vivo*. Mechanistically, Nectin-4 promoted epithelial-mesenchymal transition (EMT) by activating PI3K/AKT/NF-κB signaling through down-regulation of miR-520c-3p.

Conclusions

This study firstly clarified that Nectin-4 promoted OS progression and metastasis through activating PI3K/AKT/NF-κB signaling mediated by negative regulation of miR-520c-3p, which would open a novel avenue for identifying potential therapeutic target to improve patient outcomes.

Introduction

Osteosarcoma (OS) is now recognized as one of the most common malignant bone tumors preferentially developing in juvenile (10 ~ 30 years old), which has a high propensity for tumor recurrence and early metastasis (Smeland et al. 2019; Roberts et al. 2019). Under the current multi-disciplinary therapy situation, the 5-year survival rate of OS patients increases by 50 ~ 60% compared with surgical treatment

alone (Jafari et al. 2020; Yu et al. 2015; Xu et al. 2019; Gianferante et al. 2017). Unfortunately, numerous patients still die as a result of the fast disease progression and intense tumor invasion (Xu et al. 2019). Lung metastasis is one of commonest reasons of OS-related deaths, patients with lung metastasis take more risks of a shorter 5-year event-free survival which drop from 60–25% (Casali et al. 2018; Gao et al. 2020; Zhu et al. 2014; Liu et al. 2020). Therefore, more in-depth insight into molecular mechanisms of OS, and seeking novel molecular biomarkers and therapeutic targets are of great significance (Zhu et al. 2017; Zou et al. 2018; Wu et al. 2020).

It is commonly recognized that the disorder of cell-cell adhesion has a significant influence on tumor occurrence and development (Yasumi et al. 2003; Chothia et al. 1997). The Nectin cell adhesion molecule (Nectin) family comprise Nectin-1 ~ 4, which belong to immunoglobulin-semblable transmembrane proteins involved in the Ca^{2+} independent adherens junctions (AJs) of cell-cell and played a vital role in enhancing cellular viability and movement ability (Sakisaka et al. 2007; Nakanishi et al 2004). However, numerous studies reveal that Nectin-4 is specifically up-regulated in various cancers including breast, lung, ovarian, gastric and pancreatic cancer, and its expression is closely related with tumor oncogenesis and worse prognosis of the patients (Deng et al. 2019; Takano et al. 2009; Derycke et al. 2010; Lin et al. 2019; Fabre-Lafay et al. 2007; Nishiwada et al. 2015). In addition, it has also been described that Nectin-4 could activate the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway and relate with tumor proliferation, metastasis and angiogenesis in gallbladder and gastric cancer (Zhang et al. 2016; Zhang et al. 2018). Nevertheless, we have little idea about the functional role of Nectin-4 protein in OS.

MicroRNAs (miRNAs) are a major class of short (18 ~ 25 nts), non-coding single-stranded RNAs that can directly affect gene expression and in turn regulate biological functionality (Pal et al. 2015). These miRNAs target and bind to the mRNAs' specific 3'-untranslated region (3'-UTR) and then degrade the mRNA at the posttranscriptional level (Malagobadan et al. 2020; Zhang et al. 2012). An increasing number of studies revealed that miRNAs play a vital in the tumor growth, progression, and metastasis of OS by modulating target mRNA (Zhong et al. 2018; Zhang et al. 2019; Luo et al. 2019). For instance, Zhong et al. reports that high expression of miR-1270 is highly positively correlated with poor prognosis OS patients (Zhong et al. 2018). Moreover, Zhang et al. reveals that miR-493-5p could significantly restrain tumorigenesis and metastasis of OS (Zhang et al. 2019). Recently, accumulated evidence confirmed miR-520c-3p is strongly associated with the occurrence and progression in numerous tumors (Tang et al. 2017; Li et al. 2019; Mudduluru et al. 2017). However, the functional roles of miR-520c-3p in OS remain largely unknown.

In our study, we determined that Nectin-4 protein served as a crucial oncogene regulating OS cells proliferation, migration, invasion and metastasis both *in vitro* and *in vivo*. Moreover, we also identified the molecular mechanisms that Nectin-4 activates PI3K/AKT/NF-κB signaling mediated by modulating miR-520c-3p.

Materials And Methods

Tissue samples

For purpose of detecting the Nectin-4 expression, a total number of 29 OS and 8 normal tissue samples (containing 8 pairs of samples) were obtained from OS patients who had not accepted chemotherapy and/or radiotherapy before surgical resection in Tianjin Medical University Cancer Institute and Hospital. Informed consent to participate in research was obtained. All collected tissues were stored at liquid nitrogen. Additionally, tissue microarrays (TMAs) used for immunohistochemical (IHC) staining analysis were composed of 71 samples including 1 normal bone tissue and 70 OS tissues, sampled from 2 patients with stage IA, 31 patients with stage IIA, 31 patients with stage IIB, and 6 patients with stage IVB (Bioaitech, cat.no. L714901, Xi'an, China). This study was authorized and supervised by the Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital.

Conducting subcutaneous xenograft and lung metastatic mice models

The BALB/c-nude mice (female, 4~5 weeks old) were purchased from Beijing SPF Biotechnology Co., Ltd. All animal operations and procedures were authorized by the Animal Care Committee of the Tianjin Medical University Cancer Institute and Hospital, China. For a subcutaneous xenograft model, 5×10^6 (resuspend with 100μl saline solution) of stable shCtrl/shNectin-4 143B cells were subcutaneously injected into the flank of BALB/c-nude mice (5 mice/group). In addition, the tumor growth size was observed and recorded every 3 days by means of the calipers. At last, mice were euthanized 24 days later after subcutaneous injection, and then tumors were harvested, photographed and weighted. Besides, for the lung metastatic model, 1×10^6 stable shCtrl or shNectin-4 143B cells (resuspend with 100μl saline solution) were injected into the nude mice by lateral tail vein (5 mice/group). Three weeks later after implantation, those mice were euthanized and lung tissues were removed.

To detect the effect of miR-520c-3p *in vivo*, 5×10^6 stable shNectin-4 143B cells transfected with 50nM miR-520c-3p or NC inhibitor were subcutaneously injected into the nude mice (6 mice/group). Then, 5μg of miR-520c-3p/NC inhibitor (100μl volume) was injected into the tumor on the 9, 12, and 15 days after cell inoculation (Zhang et al. 2012). The follow-up tests were carried out as described above.

Statistical analysis

Data obeyed a normal distribution were displayed with mean ± SD. The relevance between Nectin-4 and clinicopathological indicators was evaluated by $\chi 2$ test or Chi-square analysis. The correlation between Nectin-4 and miR-520c-3p was performed by spearman's analysis. Furthermore, statistical comparison and plotting of those experimental data were carried out by Student's *t*-test, the Wilcoxon signed rank test, the ANOVA analysis, the Mann-Whitney test wherever appropriate *via* SPSS software version 22.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). *P*-value of < 0.05 was regarded to be statistically significant.

Results

The Nectin-4 expression and clinical significance in OS

Nectin 1~3 were commonly enriched in normal adult tissues, while Nectin-4 was specifically up-regulated in various tumors and played significantly role in tumor occurrence and development (Deng et al. 2019, Takano et al. 2009, Derycke et al. 2010, Lin et al. 2019). Nevertheless, we had little idea about the role of Nectin-4 protein in OS. Firstly, we were eager to monitor the expression levels of Nectin-1~4 in OS tissues and adjacent normal tissues. By using RT-qPCR, we noticed that the expression of Nectin-1~3 had no significant differences between OS and normal tissues (**Fig. 1A-1C**). As expected, we found that Nectin-4 expression was markedly increased in OS tissues compared to normal tissues (**Fig. 1D**). Similarly, the significant difference in expression of Nectin-4 was also observed in 8 paired samples of OS and adjacent normal tissues both at mRNA and protein level (**Fig. 1E** and **1F**).

In order to further investigate the expression difference of Nectin-4 between OS tissues and normal tissues, and the association between Nectin-4 and clinical features, we downloaded the related gene expression profiles and clinical data from TARGET, GTEx, and GEO database. Interestingly, the mRNA level of Nectin-4 displayed obvious overexpression in OS tissues using TARGET database compared with that in the normal muscle tissue obtained from GTEx database (**Supplementary Fig. 1**). Furthermore, to evaluate the association between the gene expression of Nectin-4 and clinical features in OS, we re-assayed the data acquired from the GSE21257. The mean value of Nectin-4 mRNA expression was regarded as the threshold and then the patients were divided into two cohorts (high expression cohort and low expression cohort). As shown in **Table 1**, we found that the Nectin-4 high expression was markedly associated with tumor metastasis (P<0.05). Strikingly, we found that more patients in the high expression group (16/19, 84.2%) exhibited tumor metastasis compared with those in the low expression group (18/34, 52.9%), which implies that Nectin-4 might regulate the tumor progression in OS.

To determine the differential expression of Nectin-4 in diverse histologic stages of OS, Nectin-4 IHC was performed on an OS TMA. Overall, 48/70 (68.6%) tumors expressed Nectin-4, with 30/48 (62.5%) tumors showing high level expression and 18/48 (37.5%) low level. High expression of Nectin-4 occurred most commonly in stage IVB OS tissues (66.7%, 4/6), followed by stage IIB (51.6%, 16/31), stage IIA (32.3%, 10/31), and stage IA OS tissues (0%, 0/2), which indicate that high expression of Nectin-4 is closely correlated with higher OS stages (**Fig. 1G**).

Next, three diverse OS cell lines (MG63, U2OS and 143B) and one osteoblastic cell line (hFOB1.19) were assessed for basal expression levels of Nectin-4 by RT-qPCR and Western blotting. The results demonstrated that Nectin-4 expression in MG63, U2OS, and 143B cell lines was significantly higher than that in hFOB1.19 cell lines (**Fig. 1H** and **1I**). The highest up-regulation of Nectin-4 was found in 143B cells, followed by MG63 and U2OS cell lines. Therefore, the 143B cell line was selected for Nectin-4 knockdown, and MG63 and U2OS cell lines were selected for Nectin-4 overexpression. Thus, we concluded that Nectin-4 is highly expressed in clinical OS tissues and OS cells.

Nectin-4 promotes OS cells proliferation

To discuss whether Nectin-4 had an influence on OS cell viability, we firstly constructed and sorted out the OS cell lines with stable Nectin-4 overexpression (Nectin-4-OE) or knockdown by means of lentivirus infection. As shown in the **Fig. 2A, 2B** and **Supplementary Fig. 2A**, we validated that Nectin-4 was successfully over-expressed in Nectin-4-OE group of U2OS and MG63 cells compared with vector negative control (Vector-NC) group cells at both mRNA and protein levels. Besides, to further validate the functional roles of Nectin-4 in OS cells, we also constructed the stable Nectin-4 knockdown OS cell line. As shown in the **Fig. 2C**, using RT-qPCR, we found that Nectin-4 was significantly down-regulated at mRNA level in 143B cells infected with Lenti-shRNAs, where shNectin-4#2 showed the most potently down-regulated effect compared with shNectin-4#1 and shNectin-4#3. Moreover, we validated the result at protein levels *via* Western blotting assays (**Fig. 2D** and **Supplementary Fig. 2B**). Thus, we chose the shNectin-4#2 cells for next investigation.

Next, we investigated the function of Nectin-4 on the cell proliferation *via* CCK-8 and colony formation assays. As presented in **Fig. 2E** and **2F**, in comparison with the Vector-NC groups, up-regulation of Nectin-4 remarkably enhanced cell proliferation both in U2OS and MG63 cell lines at different time points (24, 36, 48 and 72h) (each, $P<0.001$). Moreover, we detected the effect of down-regulated Nectin-4 on OS cell viability. As presented in **Fig. 2G**, the proliferation rates of shNectin-4#2 group cells were remarkably lower than that of shCtrl group cells at different time points (24, 36, 48 and 72h) through CCK-8 trial in 143B cell line ($P<0.01$, respectively). In addition, we also found that up-regulation of Nectin-4 could noticeably facilitate colony formation both in MG63 and U2OS cell lines compared with those in the Vector-NC groups, suggesting that overexpression of Nectin-4 could markedly promote the OS cell proliferation (**Fig. 2H** and **Supplementary Fig. 2C**, Both in MG63 and U2OS, $P<0.01$). Inversely, down-regulation of Nectin-4 noticeably restrained colony formation in 143B cells compared with those in the shCtrl group (**Fig. 2H** and **Supplementary Fig. 2C**, $P<0.001$). In conclusion, these above results indicated that Nectin-4 could significantly modulate the human OS cells proliferation.

Nectin-4 enhances human OS cells migration and invasion *in vitro*

As mentioned above, a close connection between Nectin-4 up-regulation and tumor metastases was confirmed in the GSE21257. To further investigate the effect of Nectin-4 on cell metastasis, we firstly adopted the transwell assays to observe whether the overexpression of Nectin-4 could enhance the migratory and invasive capacity of MG63 and U2OS cells. Transwell assay without Matrigel demonstrated that much more migrated cells tinted with dark blue were observed in the Nectin-4-OE group cells than those in the Vector-NC groups (**Fig. 3A**, in MG63, P<0.01 and in U2OS, P<0.001). Similarly, using transwell assay with Matrigel, we confirmed that the number of invasive cells in Nectin-4-OE groups were over twice as those in the control groups (**Fig. 3B**, in MG63, $P<0.001$ and in U2OS, $P<0.01$), implying that Nectin-4 could enhance the OS cell movement ability. Furthermore, we also detected the migration and invasion ability of shNectin-4#2 143B cell lines *via* transwell assays. As shown in **Fig. 3C**, knockdown of Nectin-4 remarkably restrained the movement activity of 143B cells where the migrated cells and invaded cells were markedly cut down compared with shCtrl cells ($P<0.01$ and $P<0.001$, respectively).

In addition, the wound-healing assay was also carried out to validate the function of the Nectin-4 up-regulation on the migration ability in OS cell lines. As demonstrated in **Fig. 3D** and **Supplementary Fig. 3**, the wound width of the Nectin-4-OE group was remarkably shortened than that of the Vector-NC group (in MG63, $P<0.01$ and in U2OS, $P<0.001$), confirming that up-regulation of Nectin-4 could remarkably heighten the migratory capacity of the MG63 and U2OS cells. Taken together, up-regulation of Nectin-4 promoted the cell movement activity in human OS cell lines. Moreover, we further observed the influence of Nectin-4 down-regulation on the migration activity in 143B cells. Compared with shCtrl group, shNectin-4#2 group cells presented with a much broader wound width (**Fig. 3D** and **Supplementary Fig. 3**, $P<0.001$). In a word, these results implied that Nectin-4 played important roles in modulating the human OS cell proliferation and movement activities *in vitro*.

Nectin-4 modulates epithelial-mesenchymal transition (EMT) *via* activating PI3K/AKT/NF-κB signal pathway

It had been reported that EMT is a classical progress in tumor cell metastasis, and we discovered that Nectin-4 played important roles in promoting OS cell movement activities. To further investigate if and how Nectin-4 could promote OS metastasis, we evaluated the expression changes of typical EMT markers in OS cell lines both at mRNA and protein levels. As shown in **Fig. 4A** and **4B**, we discovered that up-regulation of Nectin-4 led to a reduction of ZO-1 expression, an epithelial marker, and also notably elevated the expression of mesenchymal markers including Vimentin and N-Cadherin in U2OS and MG63 cells using RT-qPCR and Western blotting analysis, respectively. Furthermore, we also detected the upstream transcriptional regulators including Zeb1 and Slug of EMT signaling pathway. As displayed in **Fig. 4A** and **4B**, the expression of Zeb1 and Slug was much higher in the group of Nectin-4-OE cells than those in the Vector-NC group cells. Additionally, the changes of EMT-related molecule markers were also observed in 143B cells infected with lenti-shNectin-4#2. In contrast with Nectin-4 up-regulation, knockdown of Nectin-4 obviously down-regulated the expression of Vimentin and N-Cadherin, and enhanced the ZO-1 expression (**Fig. 4A** and **4B**). Similarly, EMT-related transcription factors (Zeb1 and Slug) also had the same trend with mesenchymal markers which were down-regulated when Nectin-4 was depleted (**Fig. 4A** and **4B**). With all of these results together, we confirmed that Necin-4 exerted a crucial influence on regulating EMT.

Numerous studies confirmed that Nectin-4 could facilitate tumor growth, angiogenesis, and metastasis *via* phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling cascade in breast cancer, gallbladder carcinoma, and colorectal cancer (Zhang et al. 2016, Zhang et al. 2018). To evaluate whether Nectin-4 could activate PI3K/AKT pathway in OS, we performed both GSVA analysis and GSEA analysis. As demonstrated in **Supplementary Fig. 4A**, GSVA analysis showed that PI3K pathway was markedly enriched in group of highly-expressed Nectin-4. In addition, using GSEA analysis, we also found that PI3K pathway displayed positive correlation with Nectin-4 expression in OS Target dataset (**Supplementary Fig. 4B**). To investigate whether Nectin-4 was involved in regulating OS-related EMT *via* PI3K/AKT pathway, we observed the expression levels of AKT and p-AKT both in MG63 and U2OS cells. We found that the expression of p-AKT in Nectin-4-OE group was much higher than that in the Vector-NC group. On the

contrary, knockdown of Nectin-4 obviously decreased the expression of p-AKT in OS cell lines (**Fig. 4C**). Besides, we also detected that up-regulation of p-NF-κB P65 (p-P65) in Nectin-4-OE group compared with Vector-NC group (**Fig. 4C**). Based on these findings, we speculate that Nectin-4 might modulate the EMT pathway through regulating PI3K/AKT/NF-κB pathway. Then, we managed to observe if we could adopt a classical AKT inhibitor named as LY294002 to reverse the effect of Nectin-4 overexpression. In our present research, we discovered that LY294002 successfully reversed the Nectin-4 overexpression effect on the expression of markers of EMT and PI3K/AKT pathway (p-AKT) (**Fig. 4D**). Moreover, to further validate that Nectin-4 could regulate EMT markers by PI3K/AKT/NF-κB signal pathway in OS cell lines. We picked out a sort of NF-κB inhibitor (PDTC) to observe its effects on Nectin-4-OE MG63 and U2OS cells. The results demonstrated that PDTC could block the efficiency of Nectin-4 overexpression on EMT-related markers and p-P65 (**Fig. 4E**). In addition, we detected that LY294002 could reverse the Nectin-4 overexpression effect on the expression levels of p-P65 (**Fig. 4D**). Functionally, we also revealed that LY294002 reversed the function of Nectin-4 up-regulation on migratory and invasive activities in both MG63 and U2OS cell lines (**Fig. 4F**, both $P<0.01$). Interestingly, we observed that the high levels of AKT and P65 expression when Nectin-4 was up-regulated, while down-regulation of Nectin-4 resulted in the opposite effect. Above all, we speculated that Nectin-4 could modulate the levels of AKT and p-65 to stimulate the PI3K/AKT/NF-κB signaling.

MiR-520c-3p is a downstream regulator that targets AKT1 and p65

Next, we were eager to clarify the deeper mechanism under which Nectin-4 modulated PI3K/AKT/NF-κB signal pathway. Given that miRNAs could play significant regulatory roles by targeting mRNAs for cleavage or translational repression, we focused on the effects of Nectin-4-regulated miRNAs that both targeted AKT and NF-κB. As AKT1 played crucial roles in PI3K/AKT signaling in OS, p65 served as the most important subunit of NF-κB complex (Zhu et al. 2014, Liu et al. 2020, Zhu et al. 2017, Zou et al. 2018). Potential miRNA targeting AKT1 and P65 were predicted by six online bioinformatics analysis software databases including TargetScan, miRanda, microT, miRmap, RNA22 and PITA (https://starbase.sysu.edu.cn/agoClipRNA.php?source=mRNA). After initial evaluation, as shown in **Fig. 5A** and **5B**, a total of 82 and 59 miRNAs targeting AKT1 and P65 were selected, respectively. Subsequently, we screened out 28 miRNAs simultaneously targeting AKT1 and p65 which were generated by taking intersection of the two miRNAs clusters using Venn diagrams (**Fig. 5C**). Among them, we selected 12 miRNAs simultaneously predicted by at least three databases (**Supplementary Table 1**). Finally, we picked out 8 miRNAs (miR-302d-3p, miR-520d-3p, miR-302c-3p, miR-302b-3p, miR-520c-3p, miR-520a-3p, miR-520b, miR-302e) with relatively high reliability (context++ score□-0.15 and weighted context++ sco □-0.15 □ in TargetScan software.

To assess which miRNA is the downstream regulator of Nectin-4, we evaluated these miRNAs expression levels by using RT-qPCR in stable Nectin-4 knockdown 143B cells. The results showed that miR-520c-3p was the microRNA with the most significantly up-regulated expression (≥2-fold) in Nectin-4 knockdown 143B cells (**Fig. 5D**). Moreover, the results of spearman's correlation analysis revealed that Nectin-4 expression was significantly negatively correlated with the expression of miR-520c-3p (**Fig. 5E)**, implying

that miR-520c-3p is a downstream regulator of Nectin-4. Besides, the negative correlation between Nectin-4 and miR-520c-3p expression was also confirmed in TARGET database (**Supplementary Fig. 5A**). In addition, we also detected the miR-502c-3p expression in 29 OS specimens and 8 adjacent normal specimens by using RT-qPCR. Both in paired and unpaired specimens, the results revealed that the miR-520c-3p was apparently lower in OS specimens compared with adjacent normal specimens (**Supplementary Fig. 5B** and **S5C**). Furthermore, we attempted to analyze the difference in miR-520c-3p expression between primary and metastatic OS samples in TARGET database. We found that the expression of miR-520c-3p was significantly lower in OS metastatic samples than primary samples (**Fig. 5F**). Therefore, we proposed that miR-520c-3p was the downstream regulator of Nectin-4, which thus was selected for follow-up studies.

Nectin-4 activates PI3K/AKT/NF-κB signaling through modulating miR-520c-3p

According to the prediction analysis by TargetScan, we confirmed the binding sites of miR-520c-3p in the 3'-UTR of AKT1 and P65 mRNA (**Fig. 6A**). The luciferase reporters were co-transfected with miR-520c-sp mimic or inhibitor into HEK-293T cells. We found that miR-520c-3p up-regulation significantly suppressed the luciferase activity of AKT1 and P65 with Wt 3'-UTRs, while down-regulation of miR-520c-3p resulted in the opposite effect. However, the miR-520c-3p mimics or inhibitor had no effect on those with Mt 3'-UTRs (**Fig. 6B** and **Supplementary Fig. 6A**). To clarify miR-520c-3p function in OS, we firstly assessed the basal expression of miR-520c-3p in different OS cell lines and osteoblastic cell line by RT-qPCR (**Supplementary Fig. 6B**). Subsequently, the 143B cells (lowest levels of miR-520c-3p) were transfected with miR-520c-3p mimics (miR mimics). And the MG63 and U2OS cells (higher levels of miR-520c-3p) were transfected with miR-520c-3p inhibitor (miR inhibitor). We identified the transduction efficiencies in the OS cell lines by using RT-qPCR (**Supplementary Fig. 6C**). Moreover, we observed miR-520c-3p overexpression could significantly reduce the expression of AKT1, p-AKT1, P65, and p-P65 in 143B cells by using Western blotting and RT-qPCR. While miR-520c-3p knockdown resulted in the opposite results in the MG63 and U2OS cells (**Fig. 6C** and **6D**). In summary, these results demonstrated that AKT1 and P65 are direct targets of miR-520c-3p in OS cells. As mentioned above, down-regulation of miR-520c-3p was closely related to OS metastases. To investigate the effect of miR-520c-3p on OS cell metastasis, we firstly detected the functional role of miR-520c-3p on the migratory capacity of OS cells by transwell assays. The results revealed that much fewer migrated cells were observed in the miR mimics group cells than those in the NC mimics groups. Conversely, the migratory capacity in miR-520c-3p inhibitor group cells was remarkably improved compared with the NC inhibitor group (**Fig. 6E** and **Supplementary Fig. 6D**). Then, to further clarify whether Nectin-4 could activate PI3K/AKT/NF-κB signaling mediated by miR-520c-3p in OS cells, miR mimics or miR inhibitor were respectively transfected into Nectin-4-OE U2OS cells and shNectin-4 143B cells, respectively. The results of Western blotting trials demonstrated that the up-regulation of miR-520c-3p successfully reversed the Nectin-4 overexpression effect on the expression of markers of EMT (Vimentin and Zo-1) and PI3K/AKT/NF-κB pathway (AKT1, p-AKT1, P65, and p-P65) (**Fig. 6F**). On the contrary, the miR-520c-3p silencing reversed the Nectin-4 knockdown effect on the expression of markers of EMT and PI3K/AKT/NF-κB pathway (**Fig. 6F**). Consistently, the transwell trials also showed that miR-520c-3p overexpression reversed the function of Nectin-4 up-regulation on migratory activities in

U2OS cells. And the knockdown of miR-520c-3p could also reverse the effect of Nectin-4 silencing on migratory ability in 143B cells (**Fig. 6G** and **Supplementary Fig. 6E**). Given these findings, we validated that Nectin-4 could promote OS cells EMT and migration by activating PI3K/AKT/NF-κB signaling mediated by miR-520c-3p.

Nectin4 enhances OS cells tumorigenesis and lung metastasis *in vivo*

To directly evaluate the role of Nectin-4 in OS cells tumorigenesis and growth *in vivo*, the subcutaneous transplantation model of human OS cells in BALB/c-nude mouse was adopted. Briefly, 143B cells infected with shNectin-4#2 or shCtrl lenti-virus were injected subcutaneously into each flank of BALB/c-nude mice. Finally, all of the mice were killed to harvest the xenograft. The results indicated that the capacity of tumorigenesis in shNectin-4#2 mice group was remarkably lower than that in the shCtrl group (P<0.01, **Fig. 7A**). Tumor growth of the shNectin-4#2 group was slower than that in the group (**Fig. 7B**). Moreover, it was obvious that the mean weight of the subcutaneous tumors generated from the Nectin-4 down-regulation group was significantly lower compared with the control group (P<0.01, **Fig. 7C**). The total protein was extracted from the tumors, and the expression of Vimentin, Ki67 and Nectin-4 were remarkedly reduced in the shNectin-4#2 group than the shCtrl group, while the expression of ZO-1 was opposite (**Fig. 7D**). Additionally, HE staining for the tumor sections was performed to evaluate the tissue morphology, which obviously revealed that much more cells nodules and masses in the shCtrl group than those in shNectin-4#2 group (**Fig. 7E**). Meanwhile, the IHC staining also provided the same powerful evidence that the expression of Ki-67, Nectin-4 and Vimentin in Nectin-4 knockdown group were significantly decreased compared with that in control group, also an opposite trend to the expression of ZO-1 (**Fig. 7E**). Given these findings, we identified that the knockdown of Nectin-4 could markedly attenuate the OS cells tumorigenesis and growth *in vivo*.

Then, to further verify the crucial effect of Nectin-4 on the process of OS metastasis, we also conducted a lung metastatic mouse model. Briefly, shNectin-4#2 or shCtrl 143B cells were injected into the five BALB/c-nude mice by lateral tail vein, respectively. After 3 weeks, the mice were anaesthetized, and their lungs were dissected. As shown in **Fig. 7F** and **7G**, the quantities and incidence of pulmonary metastasis in shNectin-4#2 group mice were fewer than those in shCtrl group through visual observation. Importantly, the number of OS metastasis cells with dark stained of nucleus in shNectin-4#2 group were less than that of in the shCtrl group using HE staining (**Fig. 7H**). Collectively, these data clearly indicated that knockdown of Nectin-4 could prominently restrain the tumorigenesis and metastatic of OS *in vivo*.

To investigate whether Nectin-4 promotes tumorigenesis mediated by miR-520c-3p *in vivo*, we detected the function role of miR-520c-3p knockdown in tumorigenesis on the basis of Nectin-4 silencing. Firstly, the shNectin-4#2 143B cells were transfected with miR-520c-3p inhibitor or NC inhibitor and then subcutaneously injected into the flank of six nude mice. Finally, the tumors were harvested from mice at 24 days after injection. The tumors derived from the group of "shNectin-4#2+miR inhibitor" were much larger in weight and volume than those from the group of "shNectin-4#2+NC inhibitor" (**Fig. 7I and Supplementary Fig. 7**). The results demonstrated that miR-520c-3p silencing significantly reversed the

effect in tumorigenesis and growth caused by Nectin-4 silencing. Given these findings, we confirmed that Nectin-4 and miR-520c-3p played a vital role in promoting the proliferation and metastasis of OS *in vivo*.

Discussion

Nectin-4 is a member of the Nectin family containing four Ca^{2+}-independent immunoglobulin-like cell adhesion molecules, and Nectin-4 plays key roles in modulating cellular viability and movement capacity (Sakisaka et al. 2007; Nakanishi et al 2004). Importantly, an increasing number of studies demonstrated that Nectin-4 is specifically overexpressed in various cancers (Derycke et al. 2010; Lin et al. 2019; Fabre-Lafay et al. 2007). For example, Challita-Eid collected more than two thousand tumor samples containing head/neck, lung, bladder, breast, pancreatic, ovarian, and esophageal tumors, where two-thirds of all specimens detected obviously high expression of Nectin-4 by IHC stain (Challita-Eid et al. 2016). Moreover, a large number of studies demonstrated that overexpression of Nectin-4 served as a tumor-associated inducer in various malignant tumors including colorectal, lung, pancreatic, ovarian and breast cancers (Deng et al. 2019; Takano et al. 2009; Derycke et al. 2010; Lin et al. 2019; Fabre-Lafay et al. 2007; Nishiwada et al. 2015). Until now, we knew little about whether or not Nectin-4 exert vital influence on OS oncogenesis and development. Thereby, this study was carried out for the purpose of investigating the functional role and molecular mechanisms of Nectin-4 in human OS cells.

In our study, we firstly found that the mRNA and protein expression level of Nectin-4 displayed significant overexpression in OS tissues compared with adjacent normal tissues. In addition, we also revealed that the Nectin-4 high expression was markedly associated with tumor metastases in GSE21257. Moreover, the IHC results of OS TMA demonstrated that high expression of Nectin-4 is closely correlated with higher OS stage. Unfortunately, the critical connection between Nectin-4 and clinical factors was not found in the TARGET-OS (**Supplementary Table 2**), which perhaps dues to the limited OS tumor specimens. Hence, additional studies with a large number of samples are essential to verify the connection between Nectin-4 and clinical factors in OS. Even so, numerous studies revealed that up-regulation of Nectin-4 correlated with the tumor progression and worse prognosis in various cancer (Athanassiadou et al. 2011; Ma et al. 2016; M-Rabet et al. 2016). Athanassiadou et al. reported that overexpression of Nectin-4 had a correlation with tumor size, grade, and lymph nodes infiltration in breast cancer (Athanassiadou et al. 2011). In the study by Ma, up-regulation of Nectin-4 had a correlation with TNM stage, tumor size, tumor spread and metastasis, and vascular involvement in hepatocellular carcinoma (Ma et al. 2016). In addition, M-Rabet et al. demonstrated that patients who had higher expression of Nectin-4 were more likely to suffer a shorter life compared with those with down-regulation of Nectin-4 in triple negative breast cancer (TNBC) (M-Rabet et al. 2016). Similarly, Takano et al. reported that nearly two-thirds of patients presented with up-regulation of Nectin-4 and had a very poor survival in lung cancer (Takano et al. 2009).

Using some *in vitro* assays, we concluded that up-regulation of Nectin-4 promoted human OS cells proliferation, migration and invasion. In contrast with Nectin-4 high expression, the low expression of Nectin-4 exactly reversed the above effect *in vitro*. Similar results were also found in the study by Das et

al, in which they demonstrated that colorectal cancer (CRC) cells with Nectin-4 overexpression could facilitate the cells proliferation and movement ability, further enhance the resistance to chemoradiotherapy (Das et al. 2018). Potential mechanism underlying Nectin-4 promoted the tumor cells growth, proliferation and movement were confirmed by regulating Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling activity (Bousquet et al. 2016). Rac1, as one of a member of the Rho family GTPases, exerted great influence on tumor occurrence and development (Karlsson et al. 2009). Rac1 GTPase switched Rac1-GDP (“OFF” state) to a Rac1-GTP (“ON” state) (Schmidt et al. 2002; Guo et al. 2015). In addition, several researches reported that elevated levels of Rac1 could be activated by upstream modulator of PI3K/AKT in gallbladder carcinoma, gastric cancer and breast cancer (Siddharth et al. 2017; Henderson et al. 2015).

It was reported that EMT was the most critical cellular event before the occurrence of tumor migration, invasion and metastasis (Slabáková E et al. 2011). Recent studies reported that Nectin-4 firstly combined with afadin and then regulated the actin cytoskeleton remodeling which could induce EMT and enhance pseudopod driving force in tumor cell lines (Dibyendu et al. 2015; Shankar et al. 2015). In the recent research by Hao et al, down-regulation of Nectin-4 in Papillary thyroid cancer (PTC) cells suppressed EMT and markedly inhibited PTC cell migration and invasion *via* PI3K/AKT signal pathway (Hao et al. 2019). In our study, we demonstrated that up-regulation of Nectin-4 could contribute to a reduction of ZO-1 expression, and notably elevated the expression of Vimentin and N-Cadherin. In contrast with Nectin-4 up-regulation, the down-regulation of Nectin-4 had the opposite effects on EMT-related markers. To investigate whether Nectin-4 could activate PI3K/AKT pathway in OS, we performed both GSVA analysis and GSEA analysis. The results revealed that PI3K pathway was markedly enriched in high expression of Nectin-4 group. Moreover, we revealed that Nectin-4-OE group cells had higher p-AKT and p-P65 expression than those in the Vector-NC group. Subsequently, we observed that AKT inhibitor (LY294002) and P65 inhibitor (PDTC) could reverse the impact of up-regulated Nectin-4 on the regulation of EMT-related markers, and migration and invasion capacities.

A growing body of research suggested that microRNAs played a pivotal role in the progression and metastasis of OS by regulating target mRNAs (Zhang et al. 2019; Chang et al. 2015; Tang et al. 2015). Therefore, we tried to find a novel miRNA to explain the underlying molecular mechanisms of OS development and progression development. Next, we focused on the effects of Nectin-4-regulated miRNAs that both targeted AKT and NF-κB. In our study, 8 potential miRNAs with relatively high reliability simultaneously targeting AKT1 and p65 were firstly selected by using six online bioinformatics analysis software databases. More importantly, miR-520c-3p was the microRNA with the most significantly up-regulated expression (≥ 2-fold) in stable Nectin-4 knockdown 143B cells by RT-qPCR. Subsequently, we observed that miR-520c-3p mimics and miR-520c-3p inhibitor could reverse the impact of Nectin-4 overexpression and silencing on the regulation of EMT-related markers, and migration capacities, respectively. In addition, the luciferase reporter assay confirmed that miR-520c-3p could directly target AKT1 and p65. Given these findings, we validated that Nectin-4 could promote OS cells EMT and migration by activating PI3K/AKT/NF-κB signaling mediated by miR-520c-3p (Fig. 8). In recently study, miR-520c-3p was differentially expressed in numerous cancers including breast cancer, lung cancer and

colorectal cancer and its expression was closely associated with cancer progression and prognosis in patients (Tang et al. 2017; Li et al. 2019; Mudduluru et al. 2017; Li et al. 2020). Similar results were also found in our study, the expression of miR-520c-3p was significantly lower in OS tissues than normal tissues and its low expression was positively associated with the tumor metastasis.

At last, we had successfully built subcutaneous transplantation model and lung metastatic mouse model. Of note was the results that clearly indicated that knockdown of Nectin-4 could prominently reduce the tumorigenesis and metastasis of OS cells in vivo. Besides, we also found that miR-520c-3p silencing significantly reversed the effect in tumorigenesis and growth caused by Nectin-4 silencing. Based on the above results, we confirmed that Nectin-4 promoted OS occurrence and development through activating PI3K/AKT/NF-κB signaling by down-regulation of miR-520c-3p.

Conclusion

Taken together, our present study firstly demonstrated the clear evidences that Nectin-4 was specifically up-regulated in OS tissues and cells, and its expression was closely related with tumor stage and metastasis. In addition, Nectin-4 promoted OS progression and metastasis by activating PI3K/AKT/NF-κB signaling mediated by miR-520c-3p. Therefore, there was no doubt that the potential association between Nectin-4 and OS would open a novel avenue for identifying potential therapeutic target to improve patient outcomes.

Declarations

Author contributions

GW, JF and LL conceptualized and designed this study. YL, GL, YTZ and XYH conducted the experiments. YL and YZ wrote the manuscript. GL performed bioinformatics analysis. YX, JZ, XW and YS collected and analyzed the relevant data. ZZ, XH, CZ and JZ designed and finalized the tables and figs. CZ and YM reviewed and corrected the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data presented are provided freely in this manuscript including any supplementary data.

Compliance with ethical standards

Conflict of interest statement

The authors declare no competing interests.

Ethics approval

This study was authorized and supervised by the Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital.

Consent to participate

All patients in the study were from Tianjin Medical University Cancer Institute and Hospital, and informed consent to participate in research was all obtained.

Consent for publication

All authors were agreed with publication.

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Figures

Figure 1

The Nectin-4 expression and clinical significance in OS. (**A-D**) The expression of Nectin-1~4 in in OS (n=29) and adjacent normal tissues (n=8) by using RT-qPCR analysis. (**E-F**) The expression of Nectin-4 in the 8 paired samples of OS and adjacent normal tissues at mRNA and protein level. (**G**) Immunohistochemical (IHC) stain analysis of Nectin-4 expression in OS specimens and normal tissues on tissue microarrays (scale bars 100μm, magnifications of 200×). (**H, I**) The basal expression levels of Nectin-4 in OS cell lines (MG63, U2OS and 143B) and osteoblastic cell line (hFOB1.19) by using RT-qPCR and Western blotting. ns, no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 2

The effect of Nectin-4 on human OS cells proliferation. (**A** and **B**) The expression of Nectin-4 in MG63 and U2OS cells infected with vector negative control (Vector-NC) or Nectin-4 overexpression (Nectin-4-OE) lenti-virus was verified by RT-qPCR analysis and Western blotting assay, respectively. (**C** and **D**) The effectiveness of shNectin-4#1, #2, and #3 at mRNA level in 143B cells was verified by RT-qPCR and Western blotting, respectively. (**E** and **F**) The cell proliferation curve of MG63 and U2OS cells infected with Vector-NC and Nectin-4-OE lenti-virus *via* CCK-8 assay. (**G**) The cell proliferation curve of 143B cells in shCtrl group and shNectin-4#2 group using CCK-8 assay. (**H**) Colony formation of MG63, U2OS cells (infected with Vector-NC or Nectin-4-OE lenti-virus), and 143B cells (infected with shCtrl or shNectin-4#2 lenti-virus). Each assay was duplicated for at least three times. ns, no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 3

The function of Nectin-4 on human OS cells migration and invasion. (**A**) The migration ability of the MG63 and U2OS cells with lenti-Nectin-4 or lenti-Vector-NC infection was assessed using transwell assay without Matrigel (scale bars 500μm, magnifications of 100×). (**B**) The invasion ability of MG63 and U2OS cells in Vector-NC group and Nectin-4-OE group *via* transwell assay with Matrigel (scale bars 500μm, magnifications of 100×). (**C**) The comparation of migration and invasion abilities of the 143B cells in shCtrl group and shNectin-4#2 group *via* transwell assay with or without Matrigel (scale bars 500μm, magnifications of 100×). (**D**) Wound healing assay was performed to measure the migration capacity of the MG63, U2OS cells (in Nectin-4-OE group and Vector-NC group), and 143B (in shNectin-4#2 group and shCtrl group), respectively (scale bars 100μm, magnifications of 40×). Each assay was duplicated for at least three times. ns, no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

Figure 4

Nectin-4 modulates EMT and migration potency via PI3K/AKT/NF-κB signal pathway. (**A**) The influences of Nectin-4 on the expression of EMT-related markers in 143B, MG63 and U2OS cell lines (infected with Nectin-4-OE, Vector-NC, shCtrl and shNectin-4#2 Lentivirus, respectively) by RT-qPCR. (**B**) The effects of Nectin-4 overexpression or knockdown on the levels of EMT-related markers in cells treated as above by Western blotting. (**C**) The effects of Nectin-4 overexpression or knockdown on the levels of PI3K/AKT/NF-κB pathway-related markers in cells treated as above by Western blotting. (**D**) The protein expression levels of EMT-related and PI3K/AKT pathway-related markers in Nectin-4-OE U2OS and MG63 cell lines treated with DMSO and LY294002 (ATK inhibitor). (**E**) The protein expression levels of EMT-related and

PI3K/AKT pathway-related markers in Nectin-4-OE U2OS and MG63 cell lines treated with DMSO and PDTC (P65 inhibitor). (**F**) The migration and invasion capacity in Nectin-4-OE U2OS and MG63 cell lines treated with DMSO and LY294002 (scale bars 500μm, magnifications of 200×). The concentration of both LY294002 and PDTC was 10 mM, and dissolved in DMSO. Each assay was duplicated for at least three times. ns, no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

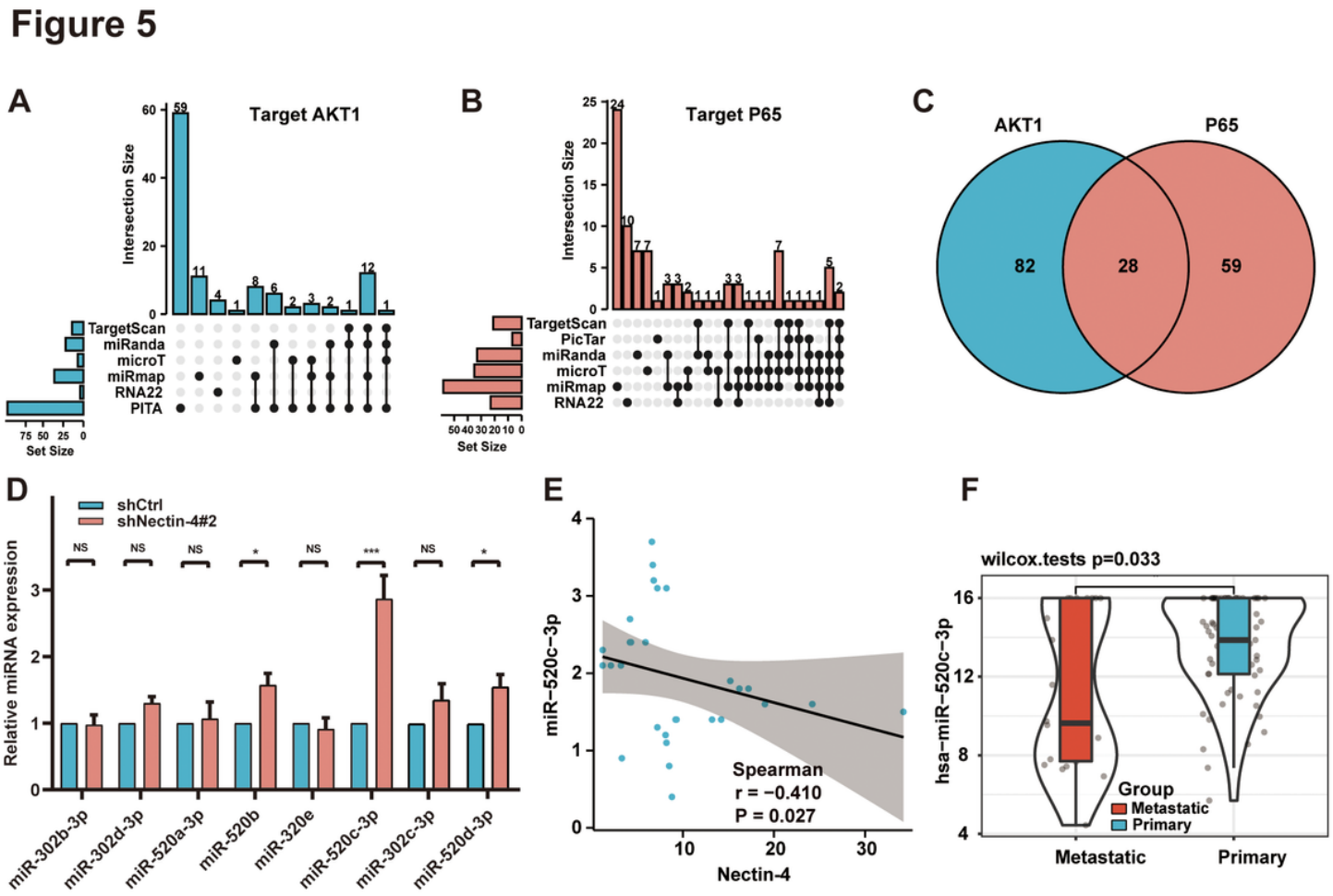

Figure 5

MiR-520c-3p is a downstream regulator that targets AKT1 and p65. (**A** and **B**) The potential miRNA which targeting AKT1 or P65 were predicted by six online bioinformatics analysis software databases including TargetScan, miRanda, microT, miRmap, RNA22 and PITA. (**C**) The miRNAs concurrent targeting AKT1 and p65 which generated by taking intersection of the two miRNAs clusters using Venn diagrams. (**D**) The evaluation of these selected miRNAs expression levels was detected by using RT-qPCR in stable Nectin-4 knockdown 143B cells. (**E**) The spearman's correlation analysis was performed to discuss the relation between Nectin-4 and miR-520c-3p expression in OS tissues. (**F**) The differential expression of miR-520c-3p between primary and metastatic OS samples in TARGET database.

Figure 6

Nectin-4 activates PI3K/AKT/NF-κB signaling through modulating miR-520c-3p. (**A**) Schematic illustration of the complementary sequence between miR-520c-3p and AKT1(or P65). (**B**) Luciferase reporter vectors containing Wt or Mt AKT1 and P65 3'-UTR were constructed and co-transfected with miR-520c-3p mimics or NC mimics into 293T cells. Luciferase reporter assays were used to determine whether miR-520c-3p directly binds to the 3'-UTR of AKT1 or P65. (**C** and **D**) The effects of miR-520c-3p overexpression or silencing on the expression of AKT, p-AKT, P65, and p-P65 in 143B, MG63 and U2OS lines by RT-qPCR and Western blotting, respectively. (**E**) The effects of miR-520c-3p overexpression or silencing on the migration ability in 143B, MG63 and U2OS cell lines by transwell trials, respectively (scale bars 500μm, magnifications of 100×). (**F**) The protein expression levels of EMT-related (Zo-1 and Vimentin) and PI3K/AKT/NF-κB pathway-related markers (AKT, p-AKT, P65 and p-P65) in Nectin-4-OE U2OS cells transfected with miR-520c-3p mimics, and in shNectin-4#2 143B cells transfected with miR-520c-3p inhibitor. (**G**) The migration ability of Nectin-4-OE U2OS cells transfected with miR-520c-3p mimics, and shNectin-4#2 143B cells transfected with miR-520c-3p inhibitor (scale bars 500μm, magnifications of 100×). miR-520c-3p mimics, miR mimics, miR-520c-3p inhibitor, miR inhibitor. Each assay was duplicated for at least three times. ns, no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

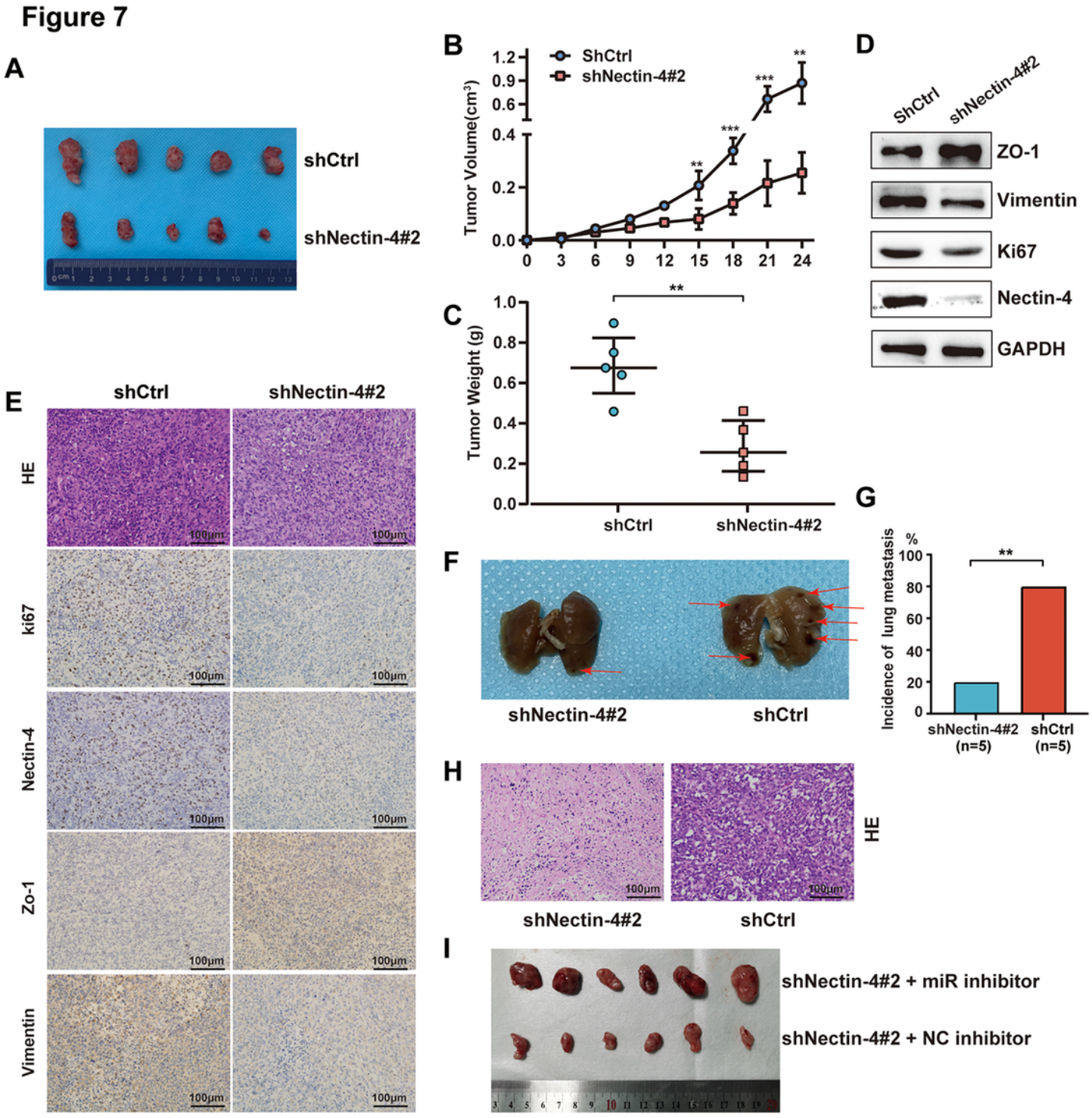

Figure 7

The effect of Nectin-4 knockdown on OS cells tumorigenesis and lung metastatic *in vivo*. (**A**) The Subcutaneous transplantation was successfully constructed after injected with shNectin-4#2 or shCtrl 143B cells. The mice were sacrificed for tumor harvesting after 24 days and tumor image. (**B**) The tumor growth curves were plotted in the shNectin-4#2 group and shCtrl group. (**C**) The weight of tumors xenografts in the shNectin-4#2 group and the shCtrl group. (**D**) The protein expression of Zo-1, Vimentin,

Ki67, Nectin-4 in the shNectin-4#2 group and the shCtrl group by using Western blotting assay. (**E**) Hematoxylin-eosin (HE) staining and IHC staining for Ki67, Nectin-4, Zo-1, and Vimentin in shNectin-4#2 group and shCtrl group (scale bars 100μm, magnifications of 200×). (**F**) Representative images of the pulmonary metastasis model in the shNectin-4#2 group and shCtrl group. (**G**) The incidence of pulmonary metastasis in the shNectin-4#2 group and shCtrl group was statistically analyzed. (**H**) The histomorphology of pulmonary tissue in the shNectin-4#2 group and the shCtrl group were performed by HE staining (scale bars 100μm, magnifications of 200×). (**I**) The Subcutaneous transplantation was successfully constructed after injected with shNectin-4#2 143B cells transfected with miR-520c-3p inhibitor or NC inhibitor. The mice were sacrificed for tumor harvesting after 24 days and tumor image. Red arrow represented the lung tissues surface metastases. Each assay was duplicated for at least three times. ns, no significance, **P*<0.05, ***P*<0.01, ****P*<0.001.

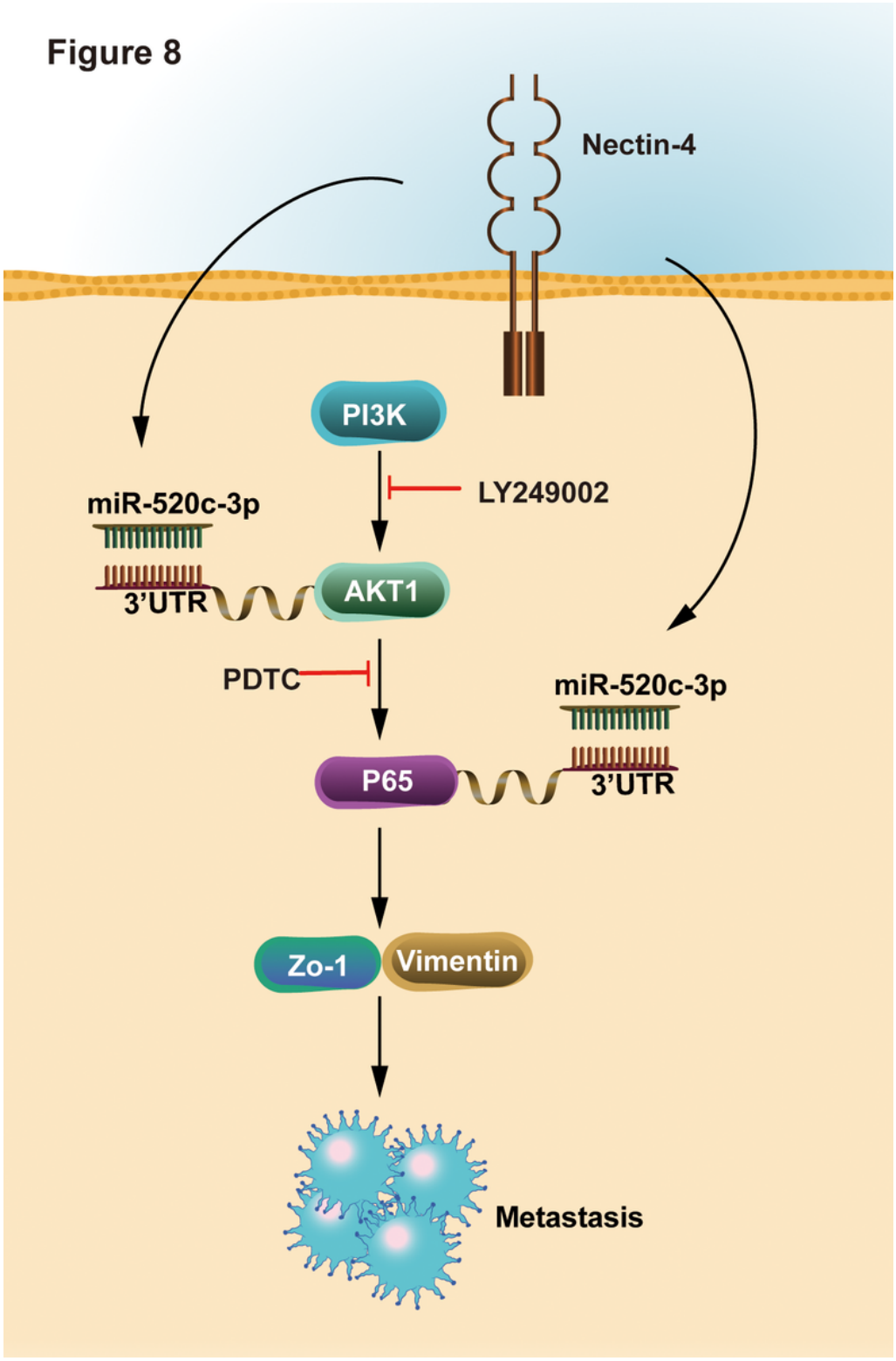

Figure 8

A proposed schematic model: the Nectin-4 regulates EMT *via* PI3K/AKT/NF-κB signal pathway mediated by miR-520c-3p.

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