

miR-204-5p Inhibits Cell Proliferation and Induces Cell Apoptosis in Esophageal Squamous Cell Carcinoma by Regulating Nestin

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Research Article

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Abstract

Purpose

This study aimed to investigate the relationship between miR-204-5p and Nestin in esophageal squamous cell carcinoma (ESCC).

Methods

The expression levels of miR-204-5p and Nestin were tested by quantitative real-time polymerase chain reaction (q-PCR) and Western blotting, respectively. The colony formation assay was used to assess cell proliferation. The flow cytometry and TUNEL assay were used to examine cell apoptosis. Tumorigenesis was evaluated using a murine xenograft tumor model.

Results

ESCC tissues and cell lines exhibited decreased miR-204-5p expression and increased Nestin expression, while the opposite results were found in paired para-carcinoma tissues and esophageal epithelial cell lines. The Luciferase reported assay confirmed that Nestin was the direct target of miR-204-5p. *In vitro*, miR-204-5p inhibited cell proliferation and induced apoptosis through regulating Nestin expression. *In vivo*, miR-204-5p inhibited xenograft tumor formation.

Conclusion

In conclusion, these results indicate that miR-204-5p inhibits cell proliferation and induces cell apoptosis in ESCC through regulating Nestin.

Introduction

Esophageal cancer (EC) is a highly malignant gastrointestinal tumor. The most common histological types of EC are esophageal adenocarcinoma (EAC) and ESCC. In Asia, especially in China, ESCC is the most common pathological subtype[1]. Even though there have been advances in diagnosis, surgical methods, radiotherapy and chemotherapy, the mortality of ESCC is still high because of the recurrence and drug resistance [2]. Advances in the study of molecular biology and gene technology may help to unravel the complicated pathogenesis of EC, which includes the interaction of genetic variations and other physiological factors. So, studying the molecular mechanisms involved in the development and progression of EC is a research hotspot, and may result in the identification of vital and novel therapeutic targets.

MicroRNAs (miRNAs) are small noncoding RNAs regulating post-transcriptional gene expression [3]. The RNA-induced silencing complex (RISC) is a target recognition element of mature miRNA [4]. The RISC binds to its target mRNA via the 3' untranslated region (UTR), and can lead to the degradation of the mRNA, or inhibition of its translation [5], both of which result in down-regulation of protein translation.

Studies have suggested that miRNAs regulate about 30% of genes, and it has been calculated that over 60% of protein-coding genes may be potential targets of miRNAs [6, 7]. The genes regulated by miRNAs are involved in numerous cellular processes, and growing evidence indicates that miRNAs participate in the pathogenesis of many diseases, including cancers, nervous system diseases, autoimmune diseases, metabolic diseases, and cardiovascular diseases [8–13]. Importantly, abnormal miRNA expression has been found to be closely related to tumorigenesis and tumor progression [14].

Nestin is an intermediate filament protein (class VI), and is expressed in normal and pathological cells of a number of different tissues and organs [15]. Recent studies have reported that Nestin is expressed in certain malignant cells, and the high Nestin expression correlates with malignant features in some tumors. Other studies have indicated that Nestin is a novel biomarker of brain, bladder, and pancreatic cancer stem cells [16–18]. To date, rare studies have revealed that Nestin positively contributes to cell proliferation and poor prognosis in esophageal squamous cancer [19]. However, the function of miRNAs on the regulation of Nestin in ESCC was unclear.

Thus, the aim of our study was to identify miRNAs targeted Nestin, determine whether these miRNAs were associated with ESCC, and uncover the mechanism how miRNAs regulate Nestin in ESCC.

Materials And Methods

Bioinformatics analysis

The *TargetScan* website and the *microRNA.org* website were searched to screen for miRNAs highly related to Nestin [20, 21]. Data from both websites were collected and filtered based on free energy and score value. The filters were designed to predict potential targets, which were then analyzed.

Tissue samples

Fresh ESCC tissue samples and para-carcinoma tissue samples were collected from 3 patients with ESCC treated in First Affiliated Hospital, Sun Yet-San University in 2018. The Institutional Ethics Committee of the First Affiliated Hospital of Sun Yet-San University approved this study.

Genetic detection

Trizol (Invitrogen, U.S.A.) was applied to extract the total RNA from the ESCC tissues according to the manufacturer's protocol. And then, the total RNA was qualified and quantified. For each sample, one microgram total RNA was prepared by library. The Agilent 2100 bioanalyzer was used to check the distribution of the size of the fragments for the library. Quantitative real-time polymerase chain reaction (qPCR, TaqMan Probe) was used to quantify the library.

The final PCR products were sequenced by the BGISEQ-500 platform (BGI-Shenzhen, China). The raw tags from the platform were processed according to the protocol, and the clean tags were obtained after filtering. The small RNA expression level was then calculated by using unique molecular identifiers [22].

Differential expression analysis was performed using the DEGseq to determine the significance of differences of expression [23].

Cell culture

ESCC cell lines of KYSE30, KYSE150, KYSE450 and a normal epithelial cell line were purchased from the Cell Bank (Shanghai, China). Cell lines were cultured according to the guideline.

Cell transfection and stable cell construction

In this study, pCDH-GFP + Puro lentiviral vectors were utilized for the stably transduced cell lines. All vectors used for the stably transduced cell lines were purchased from Hechuang Biotech Ltd. (Guangzhou, China). Nestin cDNA including 3'UTR was used in this study.

The KYSE30 and KYSE450 cell lines were transfected with different lentiviral vectors to establish blank expression vector-transfected stable cells (Blank vec group), miR-204-5p expression stable cells (miR-204-5p OE group), Nestin expression stable cells (Nestin OE group) and miRNA-204-5p expression plus Nestin expression stable cells (miR-204-5p OE + Nestin OE group), according to standard protocols. The stable cell lines were selected by measuring the expression level of GFP (Green fluorescent protein). In addition, the expression of miRNA-204-5p and Nestin in the stably transfected cells was verified by qPCR and Western blotting, respectively.

Total RNA extraction and qPCR

Total RNA from cultured cells or tissues was extracted by TRIzol reagent according to the manufacturer's instructions. For mRNA, 2 mg RNA was used for cDNA synthesis by High-Capacity RNA-to-cDNA™ Kit. For miRNA, 2 mg RNA was reverse transcribed using TaqMan miRNA assays (ABI, Forest City, CA, U.S.A.). The qPCR was performed by SYBR-based Roche Light-Cycler® 480II PCR instrument. Moreover, the relative expression levels of target genes was analyzed with $2^{-\Delta\Delta Ct}$ method [24]. In this study, U6 and GAPDH were used as the internal references. The following primer pairs were used for qPCR: miR-204-5p forward: 5'-UUCCCUUUGUCAUCCUAUGCCU-3', reverse: 5'-CTCAACTGGTGTCTGGA-3'; Nestin: forward, 5'-TGCGGGCTACTGAAAAGTTC-3'; reverse, 5'-GGCTGAGGGACATCTTGAG-3'; U6 forward: 5'-CTCGCTTCGGCAGCACACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH forward: 5'-GGGAAACTGTGGCGTGAT-3', reverse: 5'-GAGTGGGTGTCGCTGTTGA-3'.

Western blotting analysis

Total protein extracts were disposed according to the routine guideline. The membranes were blocked, incubated with rabbit polyclonal anti-Nestin antibody (19483-1-AP, Proteintech) overnight, and then treated with anti-mouse secondary antibody (Southern Biotech). An anti-GAPDH antibody (IPVH00010; MILLIPORE) was used as an internal control. Densitometric analyses of bands were performed by Image J (NIH Image, Bethesda, MD, U.S.A.), and then the quantification of protein abundance was performed by normalizing the densitometric data of target protein to that of GAPDH.

Luciferase reporter assay

The human wild-type Nestin 3'-UTR sequence or the mutated Nestin 3'-UTR sequence with the predicted target sites was amplified and subcloned into the pmirGLO Dual-Luciferasevector (HeChuang Biotech, Guangzhou, China). Kyse30 cells were seeded onto 24-well plates (5×10^5 cells/well) and co-transfected with luciferase reporter vectors (0.125 μ g) and miR-204-5p mimic (50 nM) or mimic negative control (50 nM) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. miR-204-5p mimic and NC mimic were purchased from Sango (Shanghai, China). Finally, luciferase activity was measured by the Luciferase Assay System (Promega, U.S.A.) in a bioluminescence detector (Promeg, GloMax) and were normalized by the Renilla luciferase activity according to the manufacturer's protocol.

Colony formation assay

Cell resuscitation and culture of stably transfected KYSE30 cells was performed according to the routine protocol. After trypsinization, the cells were diluted to 1×10^3 cell/ml and inoculated on the well plate. The cells were monitored daily until colony growth was observed. The number of colonies was calculated. The Enzyme-linked Spot Image Automatic Analyzer (AID, Germany) was used to scan and analyze the result.

Flow cytometric analysis

Apoptosis of the stably transfected cells was evaluated by flow cytometry (BD, Bioscience, U.S.A.) using an Annexin V-FITC Apoptosis Detection Kit (KGA106, Keygen, Jiangsu, China). CellQuest software was used to analyze the results.

TUNEL analysis

Cells were seeded onto a slide and fixed at 4°C for 25 minutes. The slides were washed with phosphate buffer saline (PBS) twice for 5 minutes, and then treated with 70% ethanol at -20°C overnight. At room temperature, 10 minutes, each sample was added by equipment buffer. The slides were incubated with TDT (terminal transferase) working solution, and then with 4', 6-diamidino-2-phenylindole (G3250, Promega, USA) according to standard protocols. The slides were then observed by fluorescence microscopy (MDI6000B, Leica, Germany). The nuclei of apoptotic cells exhibited green fluorescence, and the nuclei of all cells exhibited blue fluorescence.

Nude mice xenograft tumor model

Six-week-old male nude mice were adaptively fed for 3 days. And then, randomly, every 3 nude mice were grouped into 5 groups. Next, mice were treated with subcutaneous injection of wild type (WT) and stable transfected KYSE 30 or KYSE450 cells 3 times: 1) WT cells (NC group); 2) blank vector stable cells (Blank vec group); 3) miR-204-5p stable expression cells (miR-204-5p OE group); 4) Nestin stable expression cells (Nestin OE group); 5) miR-204-5p plus nestin stable expression cells (miR-204-5p OE + Nestin OE group). In all groups, trypsinized cells were resuspended in normal saline and 2×10^6 cells were injected each time.

The mice were observed, and tumor volume in each group was measured on day 4, 8, 12, 16, and 20 after injection. On day 21, the mice were killed by cervical dislocation, and tumor size and weight were measured. All animal experiments were conducted according to established guidelines for the use and care of laboratory animals, and the experiments were approved by the animal ethics committee of our institution.

Statistical analysis

SPSS software version 22 (IBM SPSS Inc. Chicago, IL, U.S.A.) was used to conduct the statistical analyses. Values of $P < 0.05$ were considered to be statistically significant.

Results

Identification of Nestin-related miRNAs

Potential target miRNAs related to Nestin in ESCC tissues were screened with a BGISEQ500 sequencer (BGI-Shenzhen). The results indicated there were 19 related miRNAs that were significantly differentially expressed between ESCC tissues and adjacent para-carcinoma tissues (Table 1). Among them, there were 2 downregulated miRNAs in ESCC tissues: 1) miR-204-5p: reading of para-carcinoma tissue = 54,471, reading of ESCC = 3,712, difference of reading between ESCC and para-carcinoma tissue = -50759, \log^2 ratio = -3.689; 2) novel-miR-36: reading of para-carcinoma tissue = 82, reading of ECSS = 0, difference of reading between ESCC and para-carcinoma tissue = -82, \log^2 ratio = -7.172 (Fig. 1). Moreover, results screening from 2 websites (TargetScan website and the microRNA.org website) based on the free energy and score value criteria identified 3 candidate miRNAs: miR-658, miR-211-5p, and miR-204-5p (Fig. 1). Taken together, miR-204-5p was the most potential target miRNA of Nestin.

In addition, previous studies have indicated that miR-204 suppresses cell proliferation, apoptosis, invasion and epithelial-mesenchymal transition (EMT) in ESCC [25–27]. Based on these studies, miR-204-5p was selected for this study.

Low miR-204-5p expression is inversely correlated with Nestin expression in ESCC tissues and cell lines

Nestin mRNA expression exhibited a reverse trend with miR-204-5p expression in the comparison between ESCC and para-carcinoma tissues, as well as between ESCC cells and esophageal epithelial cells. In esophageal squamous cancer tissues and cell lines, Nestin mRNA expression was increased, while miR-204-5p expression was decreased; in esophageal epithelial cells and para-carcinoma tissues, Nestin mRNA expression was decreased, while miR-204-5p expression was increased, and the differences were statistically significant (Fig. 2). Western blotting indicated that the level of Nestin protein in esophageal squamous cancerous cells and ESCC tissues was higher than in esophageal epithelial cells and para-carcinoma tissues (Fig. 3).

MiR-204-5p directly targets Nestin in ESCC

The Luciferase reporter assay was performed to investigate if miR-204-5p directly targeted Nestin in ESCC (Fig. 4). The basic group was co-transfected with pmirGLO and miR-204-5p mimic (mimic + basic group) or mimic NC (NC + basic group); Luciferase activity was not reduced in the basic group. The nestin-WT (wild-type) group was co-transfected with pmirGLO-Nestin WT and miR-204-5p mimic (mimic + WT group) or NC (NC + WT group); the Luciferase activity of the mimic + WT group was significantly reduced, indicating that miR-204-5p targeted Nestin.

The nestin-Mut1 group was co-transfected with pmirGLO-Nestin mut1 (mutant1) and miR-204-5p mimic (mimic + mutant1 group) or NC (NC + mutant1 group); Luciferase activity was not significantly different between the groups indicating that the Mut1 mutation site was the site where miR-204-5p targeted the Nestin 3'UTR. The nestin-Mut2 group was co-transfected with pmirGLO-Nestin mut2 and miR-204-5p mimic (mimic + mutant2 group) or NC (NC + mutant2 group); the Luciferase activity of the target gene was reduced, indicating that mut2 was not the site where miR-204-5p targeted the Nestin 3'UTR. Nestin-Mut1 + 2 were with co-transfected pmirGLO-Nestin mut1 + 2 and miR-204-5p mimic (mimic + nestin-Mut1 + 2 group) and NC (NC + nestin-Mut1 + 2 group); there was no significant difference in Luciferase activity between the groups, suggesting that Mut1 was the site where miR-204-5p targeted the Nestin 3'UTR.

MiR-204-5p and Nestin expression in stably transfected cells and their relations

Results of qPCR indicated that miR-204-5p and Nestin were stably expressed in KYSE30 and KYSE450 cells (Fig. 5).

In the miR-204-5p OE group, the expression of miRNA-204-5p was increased, while the expression of Nestin mRNA was not affected. In the miR-204-5p OE + Nestin OE group, miR-204-5p expression was increased, but Nestin mRNA expression was not significantly reduced, indicating that miR-204-5p could not inhibit Nestin mRNA expression.

Western blotting results indicated that the level of Nestin protein was significantly reduced in the miR-204-5p OE group but was relatively higher in the miR-204-5p OE + Nestin OE group. This result suggests that miR-204-5p inhibits Nestin protein expression in KYSE30 and KYSE450 cells, and the Nestin overexpression reverses miR-204-5p inhibition.

MiR-204-5p inhibits ESCC cell proliferation by targeting Nestin in vitro

Results of colony formation assay indicated that the number of cell clones was significantly increased in Nestin OE group, while that was dramatically reduced in the miR-204-5p OE group compared with negative control group, vector control cells, and the miR-204-5p OE + Nestin OE group (Fig. 6). These results suggested that miR-204-5p inhibited the clone formation ability of KYSE30 and KYSE450 cells, while Nestin overexpression reversed miR-204-5p inhibition.

MiR-204-5p induces cell apoptosis of ESCC by targeting Nestin in vitro

Cell apoptosis in stably transfected cells was analyzed by flow cytometry. Of the 5 groups tested both in KYSE30 and KYSE450 cells, the apoptosis rate was highest in the miR-204-5p OE group while that was lowest in Nestin OE group (Fig. 7A). Consistent with this result, the number of TUNEL-positive cells was highest in the miR-204-5p OE group while that was lowest in Nestin OE group (Fig. 7B). Moreover, Nestin overexpression could abolish the effect of miR-204-5p overexpression on apoptosis (Fig. 7A and B). These results suggested that miR-204-5p could induce apoptosis in KYSE30 and KYSE450 cells, which was attenuated by Nestin overexpression.

MiR-204-5p inhibits xenograft tumor growth in vivo

A murine xenograft tumor model was used to assess the effect of miR-204-5p on tumor growth. Tumor volume was measured at set time points after injection with stably transfected KYSE30 and KYSE450 cells. Of the 5 groups, tumor volume was the smallest in the miR-204-5p OE group while that was largest in the Nestin OE group (Fig. 8A). The mice were killed on day 20 after injection, and then tumor size and weight were measured. Of the 5 groups, the weight of the group injected with miR-204-5p overexpression stable cells was the lightest while that was heaviest in the group injected with Nestin overexpression stable cells (Fig. 8B). Furthermore, Nestin overexpression reversed the effect of miR-204-5p overexpression on suppressing the tumor growth (Fig. 8A and B). These results suggest that xenograft tumor growth is inhibited by miR-204-5p *in vivo*, and the inhibition is attenuated by Nestin overexpression.

Discussion

ESCC is a common and prevalent malignant tumor of digestive system worldwide, especially in China. The disease is associated with a poor prognosis and high mortality, and thus researches are focused on new and novel treatments. Targeted therapies have been shown to be promising for many cancers, such as lung cancer and breast cancer and so on. It is implied that studies of the pathogenic mechanisms of esophageal cancer may lead to the development of novel targeted treatments.

Nestin is originally found to be expressed in progenitor cells of the nervous system, and subsequently its expression has been found to be elevated in several different malignant tumors. Our prior study showed that Nestin is expressed in ESCC, and 32 of 93 cases of ESCC patients (34.4%) are positive for Nestin expression [19]. This finding is similar with that of Shinichiro et al who reported that Nestin is expressed in 35.5% of lung cancers. At the same time, Nestin is also detected in Eca-109 and TE-1 cells [28].

Our prior study has confirmed the association between Nestin and ESCC, and the results of that study lead us to 3 conclusions: 1) Nestin can be expressed in ESCC tissues and cell lines; 2) Nestin-positive ESCC is associated with a poor prognosis; 3) A positive correlation between Nestin phenotype and tumor cell proliferation indexes (Ki67 and PCNA) indicated that Nestin may be involved in the malignant proliferation of ESCC. However, only one study has revealed that Nestin positively contributes to cell

proliferation and poor prognosis in ESCC [19]. Thus, the current study was performed to explore the mechanism how Nestin regulates the proliferation of ESCC cells.

In recent years, several studies have revealed the associations between miRNAs and esophageal carcinoma. For example, it has been reported that miR-186 regulates cell proliferation and apoptosis in human ESCC by targeting SKP2 [29]. MiR-134 suppresses the development of ESCC through blocking MAPK pathway via targeting PLXNA1 [30]. Moreover, circular RNA ciRS-7 enhances the growth and metastasis of ESCC through inhibiting the effect of miR-7 on HOXB13 [31]. However, the role of miR-204-5p in the development of esophageal cancer is still not clear.

More and more researches reveal that miR-204-5p plays an important role in many diseases, especially in malignant tumors. Wang *et al.* investigated the expression and functional roles of miR-204-5p in OSCC [32]. They found that miR-204-5p could enhance OSCC cell proliferation and metastasis by targeting CXCR4. In addition, some studies have shown that miR-204-5p is regulated by long noncoding RNA (lncRNA). Yu *et al.* reported that lncRNA TUG1 positively regulated the expression via sponging miR-204-5p to promote osteogenic differentiation in calcific aortic valve disease (CAVD) [33]. Recently, the study of miR-204-5p in ESCC has also been reported. Tang *et al.* investigated the potential role of miR-204-5p in ESCC. They found that miR-204-5p functioned by directly targeting IL-11 [26]. However, the molecular mechanism and signal transduction mechanism of miR-204-5p in ESCC are still unknown. In the current study, we demonstrated for the first time that miR-204-5p regulates ESCC via targeting Nestin.

Our results suggest that miR-204-5p targets the Nestin 3'UTR, and the target recognition element of RISC bound to the 3'UTR of targeted mRNA. Because miRNA-204-5p is not tightly bound to Nestin, it only inhibits Nestin protein translation, but does not degrade Nestin mRNA, resulting in down-regulation of protein expression. As an upstream regulator of Nestin, miR-204-5p may inhibit cancer cell proliferation and induce cancer cell apoptosis in esophageal squamous cancer cells.

Conclusions

The results of this study showed that miR-204-5p may target Nestin mRNA, and miR-204-5p could inhibit the proliferation and induced apoptosis of ESCC cells by regulating Nestin. These results suggested that miR-204-5p maybe a key regulator of Nestin in ESCC and targeting miR-204-5p might be a novel treatment for ESCC.

Abbreviations

EC: esophageal cancer; EAC: esophageal adenocarcinoma; ESCC: esophageal squamous cell carcinoma; RISC: RNA-induced silencing complex; UTR: untranslated region; HEEC: human esophageal epithelial cell lines; qPCR: quantitative real-time polymerase chain reaction; RT-PCR: reverse transcription polymerase chain reaction; GFP: Green fluorescent protein; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; GAPDH: glyceraldehydes-3-phosphate dehydrogenase;

TUNEL: terminal deoxynucleotidyl transferase mediated nick end labeling; PBS: phosphate buffer saline; NC: negative control; PCNA: proliferating cell nuclear antigen; CDK5: cyclin dependent kinase-5.

Declarations

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Conflicts of interest

The authors have no conflict of interest.

Availability of data and material

The datasets used during this study are available from the corresponding author on request.

Code availability

Not applicable.

Author contributions

BLZ, HHL, and FFZ conceived the study. BLZ, HHL, FFZ, and WZL participated in the analysis of ESCC specimens and cell lines. BLZ, WZL, and HYZ performed qPCR, Western blotting and constructed stably transfected cell lines. BLZ, HYZ, CXL, and FL conducted the cell proliferation assay and apoptosis test. BLZ, HHL, and FFZ performed statistical analysis of all data. BLZ and HHL designed the experiments, coordinated the study, and drafted the manuscript. All authors have read and approved the final version of the manuscript.

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Figures

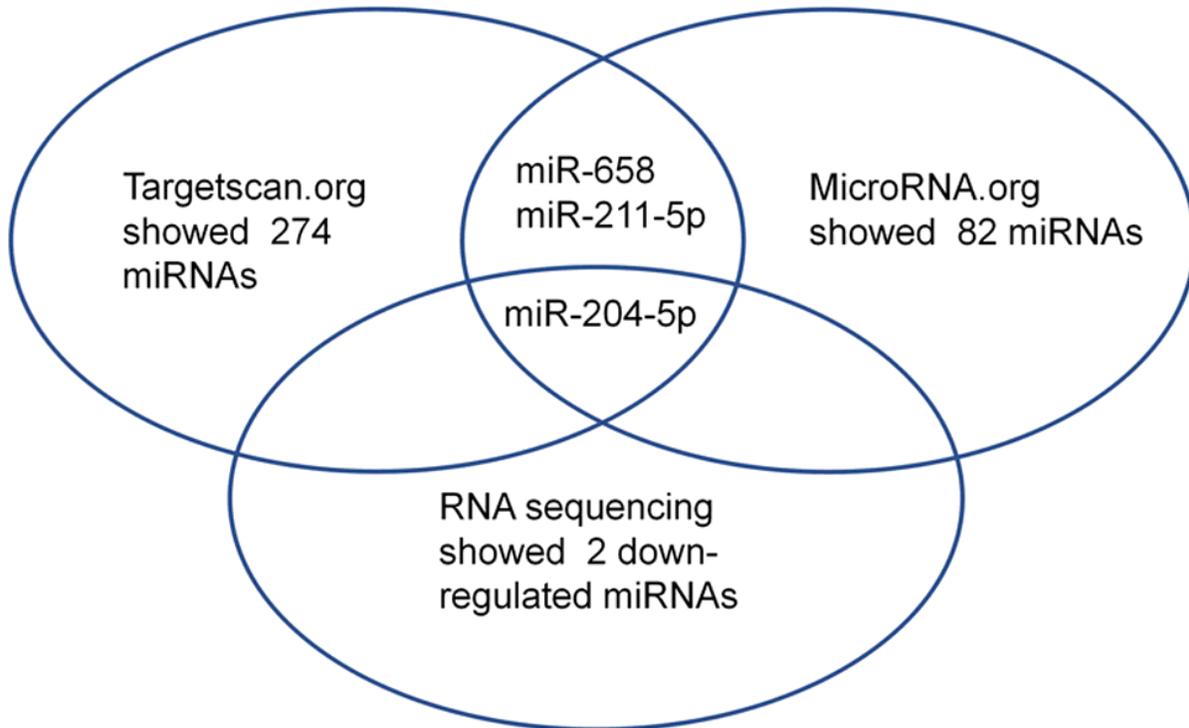


Figure 1

Among the microRNAs screened by [Http://www.targetscan.org](http://www.targetscan.org) or [Http://www.mircroRNA.org](http://www.mircroRNA.org) and RNA sequencing of ESCC, one candidate microRNA was overlapped.

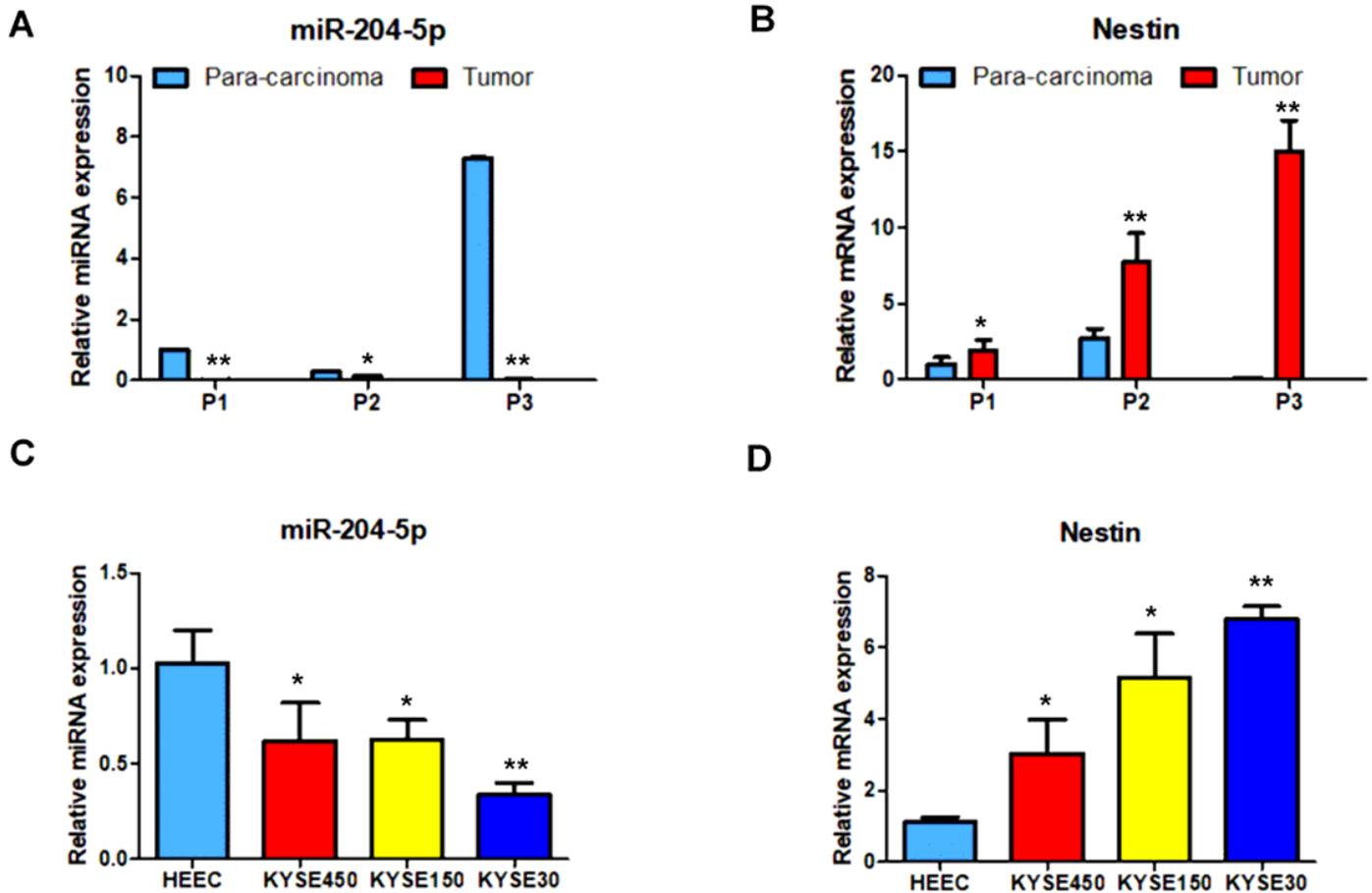


Figure 2

Expression of miR-204-5p and Nestin mRNA in ESCC tissues and para-carcinoma tissues, as well as ESCC cell lines and esophageal epithelial cell lines. A and B: The expression levels of miR-204-5p and Nestin mRNA in ESCC tissues and para-carcinoma tissues were determined by qPCR. C and D: The expression levels of miR-204-5p and Nestin mRNA in ESCC cells and esophageal epithelial cells were determined by qPCR. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ compared to control group (HEEC group or para-carcinoma group).

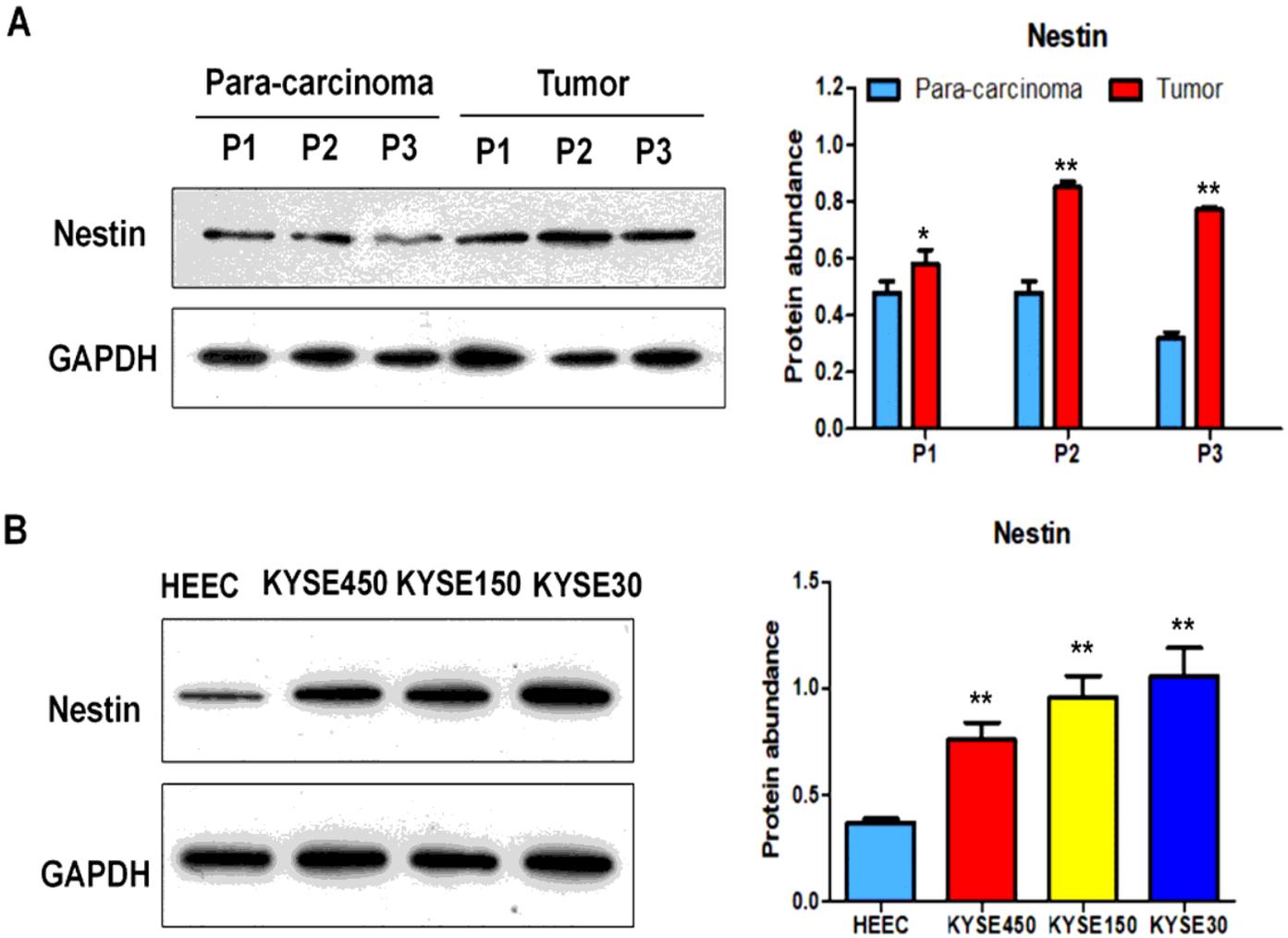


Figure 3

Protein expression of Nestin in ESCC tissues and para-carcinoma tissues, as well as ESCC cell lines and esophageal epithelial cell lines. A: The protein level of Nestin in ESCC tissues and para-carcinoma tissues was determined by Western blotting. B: The protein level of Nestin in ESCC cells and esophageal epithelial cells was determined by western blot. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ compared to control group (HEEC group or para-carcinoma group).

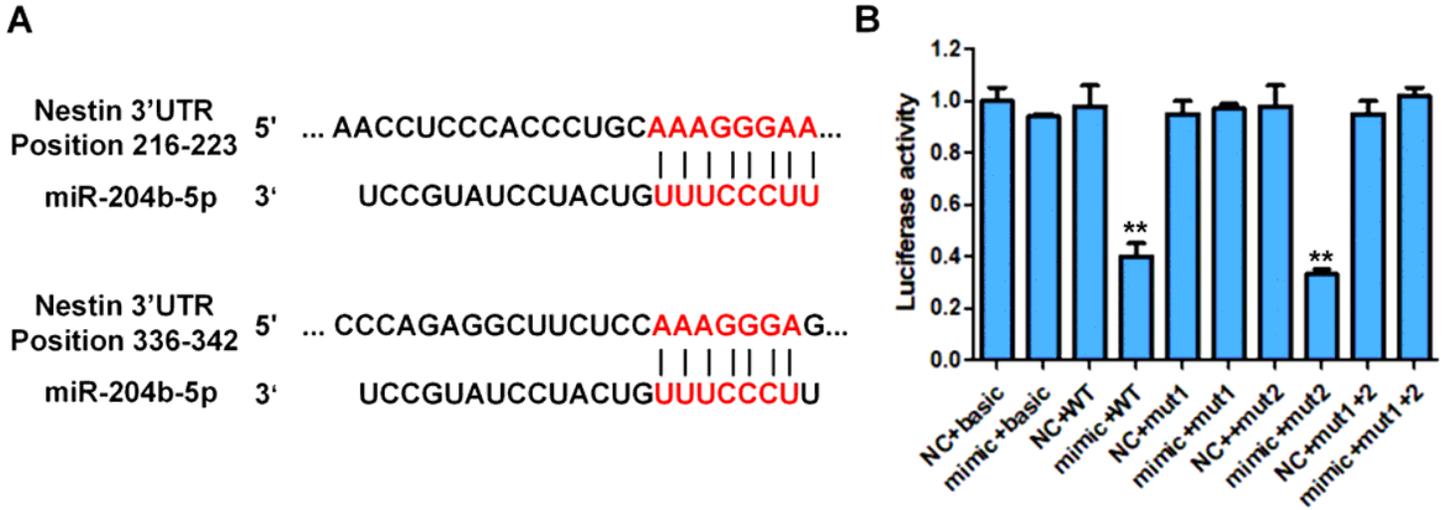


Figure 4

Luciferase activity of different groups of cotransfected plasmid cells with different plasmid cotransfected combination (pmirGLO, pmirGLO-Nestin WT, pmirGLO-Nestin mut1, pmirGLO-Nestin mut2, pmirGLO-Nestin mut1+2). A: Schematic representation of the nestin mRNA depicting miR-204-5p binding sites in its 3'-UTR. B: The basic group was cotransfected with pmirGLO and miR-204-5p mimic (mimic+basic group) or mimic NC ((NC+basic group). Nestin-WT (wild type) group was cotransfected with pmirGLO-nestin WT and microRNA-204-5p mimic (mimic+WT group) or NC (NC+WT group). Nestin-Mut1 group was cotransfected with pmirGLO-nestin mut1 (mutant1) with miR-204-5p mimic (mimic+mutant1 group) or NC (NC+mutant1 group). Nestin-mut2 group was cotransfected with pmirGLO-nestin mut2 and miR-204-5p mimic (mimic+mutant2 group) or NC (NC+mutant2 group). Nestin-Mut1+2 was with cotransfected with pmirGLO-nestin mut1+2 and miR-204-5p mimic (mimic+nestin-Mut1+2 group) and NC (NC+nestin-Mut1+2 group). *P<0.05, **P<0.01 or ***P<0.001 compared to NC group.

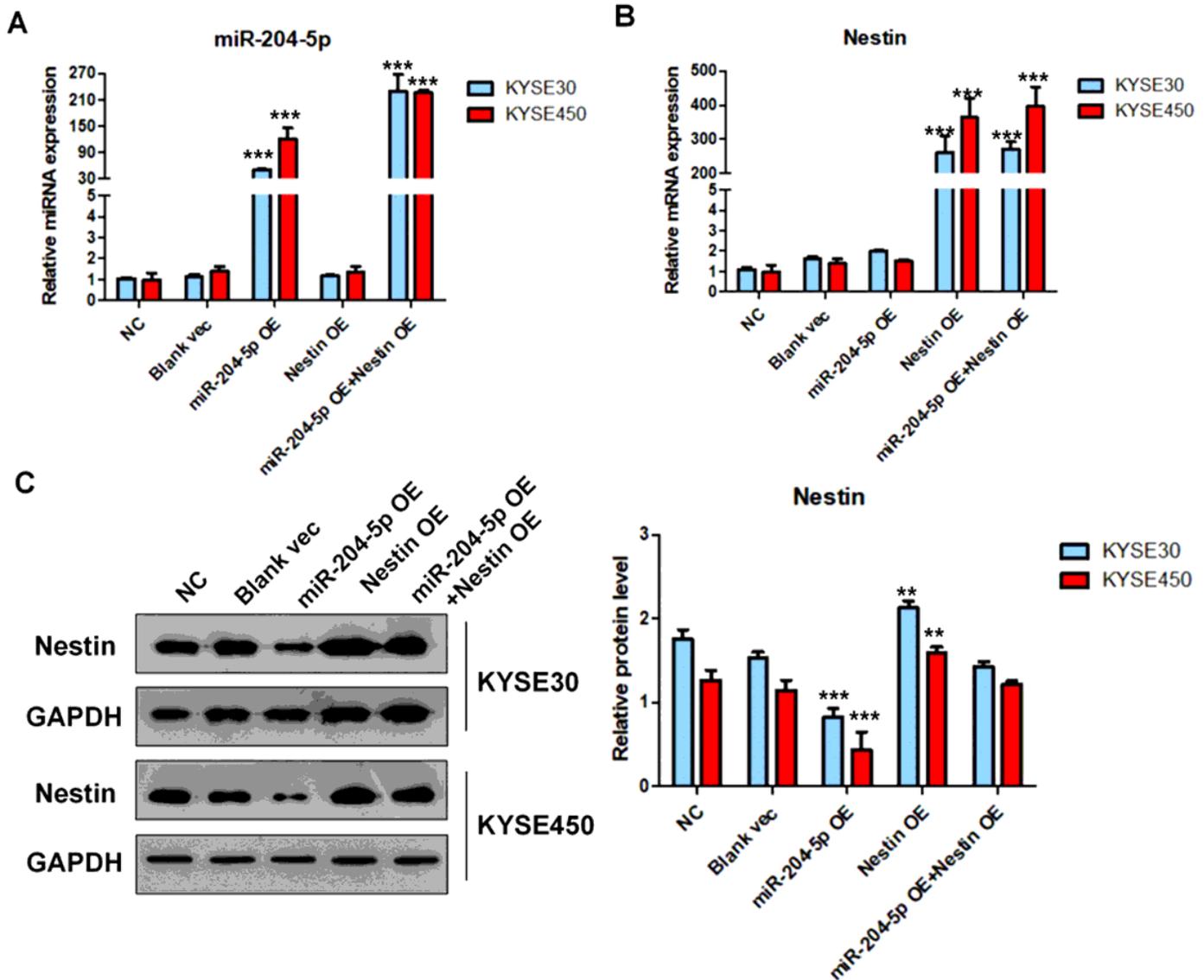


Figure 5

The expression level of miR-204-5p, Nestin mRNA and protein in KYSE30 and KYSE450 stably transfected cells, including NC group, Blank vec group, miR-204-5p OE group, Nestin OE group and miR-204-5p OE+Nestin OE group. A and B: The expression levels of miR-204-5p and Nestin mRNA in stably transfected cells were explored using qPCR. C: The protein levels of Nestin in stably transfected cells were determined by Western blotting, * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ compared to NC group.

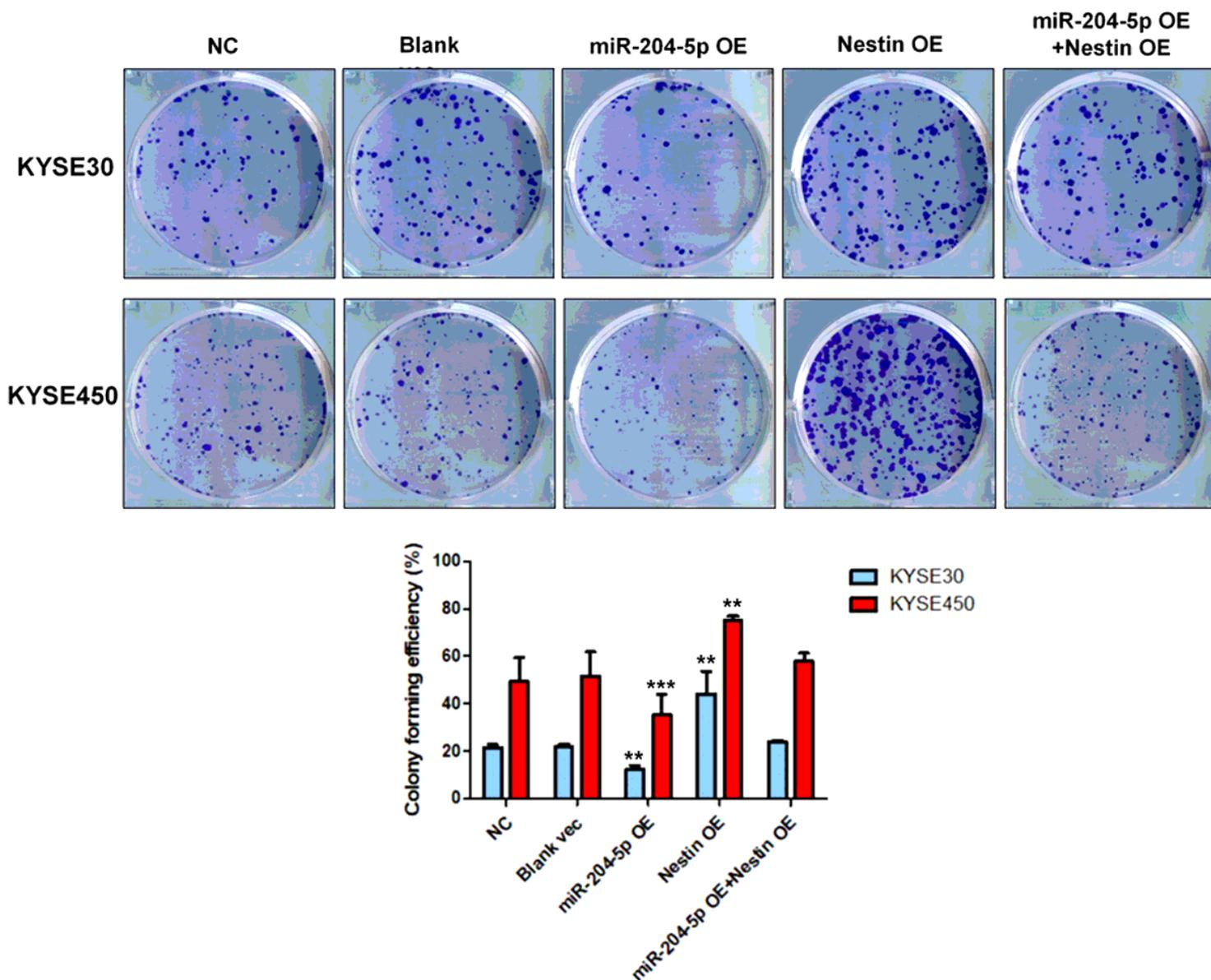


Figure 6

Colony formation assay was performed in KYSE30 and KYSE450 stably transfected cells, including NC group, Blank vec group, miR-204-5p OE group, Nestin OE group and miR-204-5p OE +Nestin OE group. *P<0.05, **P<0.01 or ***P<0.001 compared to NC group.

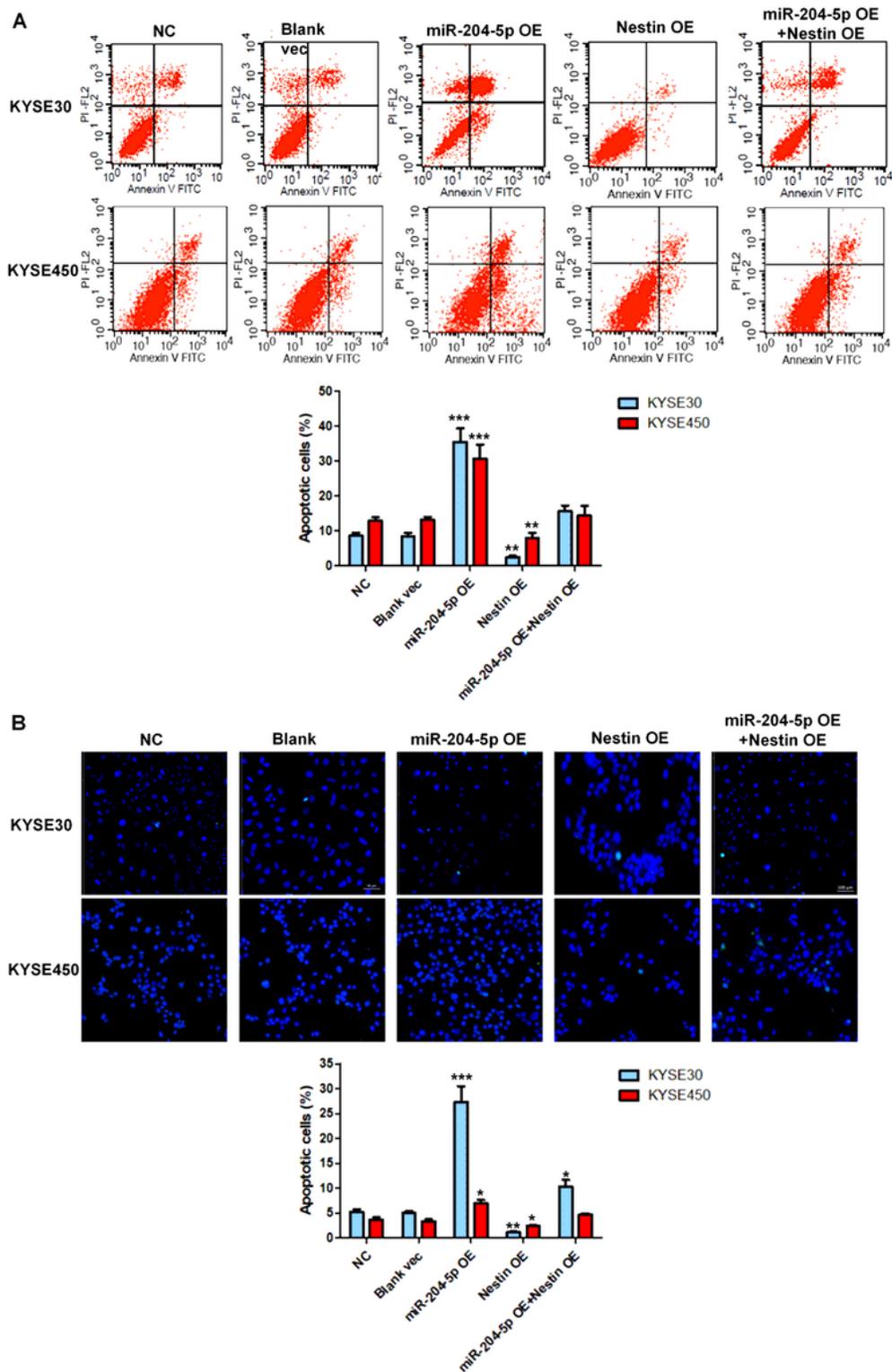


Figure 7

Cell apoptosis in the KYSE30 and KYSE450 stably transfected cells were analyzed by flow cytometry (A) and TUNEL assay (B). * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ compared to NC group.

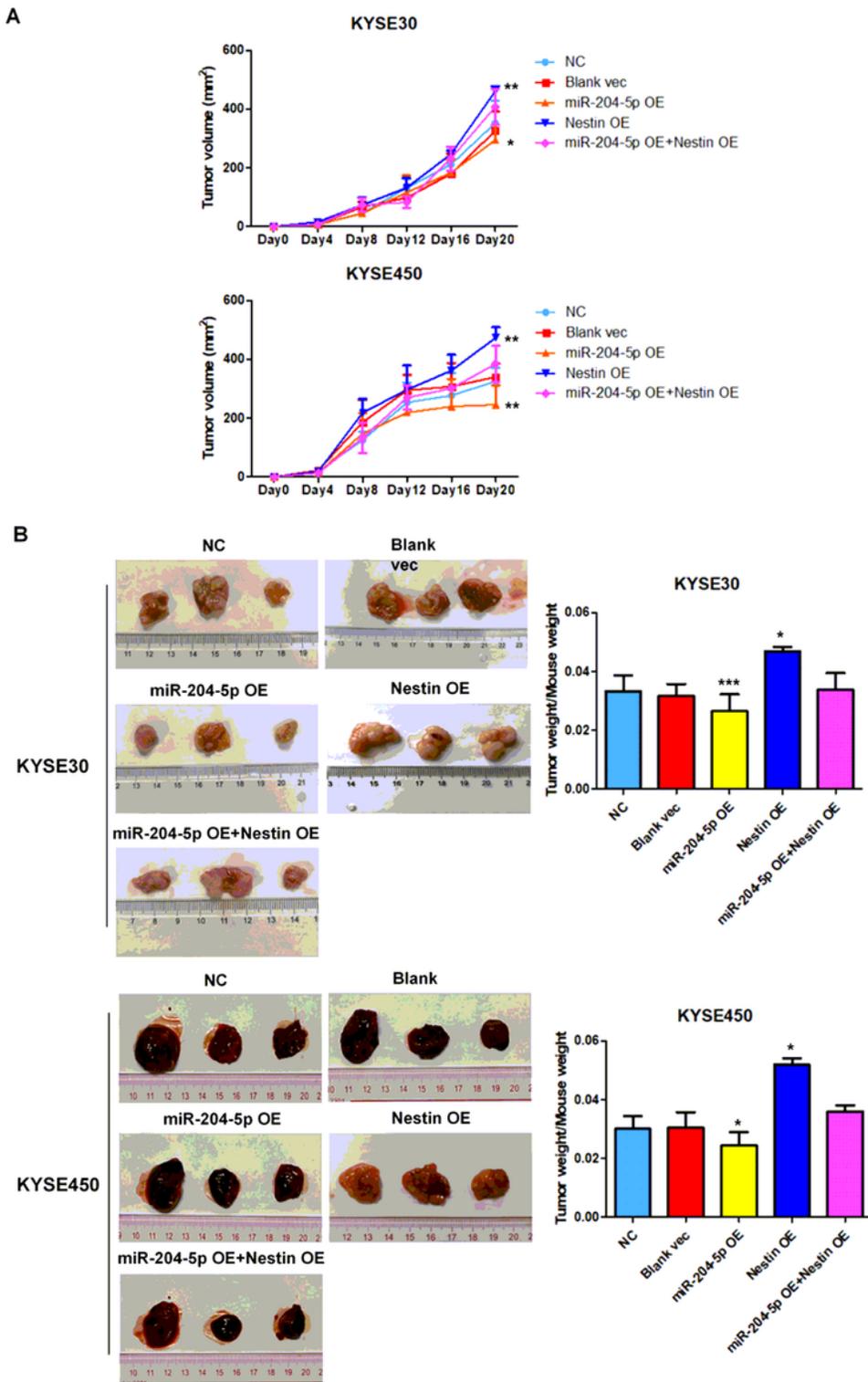


Figure 8

The effect of miR-204-5p on tumor growth was assessed by a nude mice xenograft tumor model. A: Growth curve of tumor volumes were calculated. Data were shown as mean \pm SD. B: Photographs of tumors size and weight obtained from the different groups of nude mice. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ compared to NC group.