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ORIGINAL ARTICLE

# EPHA5 enhances the stemness of non-small cell lung cancer cells through activating the Wnt signaling pathway

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## Summary

***Purpose:*** *To explore the effect of erythropoietin-producing hepatocellular receptor A5 (EPHA5) on the stemness of non-small cell lung cancer cells and its molecular mechanism.*

***Methods:*** *Highly-expressed EPHA5 in NCI-H460 and NCI-H1229 cells was silenced. After EPHA5 silencing, the positive expression level of cluster of differentiation 133 (CD133) in NCI-H460 and NCI-H1229 cells was detected by flow cytometry, and the expression levels of stemness markers sex determining region Y-box 2 (Sox2), Nanog, Kruppel-like factor 4 (KLF4) and octamer-binding transcription factor 4 (Oct4) in cells were detected by Western blotting.*

***Results:*** *The expression level of EPHA5 in non-small cell lung cancer H460 and H1229 cells was higher than that in A549 and SPC-A1 cells. After EPHA5 silencing, the levels of CD133 and stemness markers Sox2, Nanog, KLF4 and Oct4 in H460 and H1229 cells all declined. CCK-8 assay showed that Wnt agonists at a concentration of 2.5 and 5 μm had little effect on the proliferative activity of H460 and H1229 cells. Western blotting revealed that Wnt agonists at a concentration of 5 μm could better enhance the expression of β-catenin. After treatment with Wnt agonists, the expression of CD133 in H460 and H1229 cells with EPHA5 silencing by siRNA3 was higher than that before treatment, and the expression levels of Sox2, Nanog, KLF4 and Oct4 in the above two cells were also increased compared with those before treatment. However, the levels of the above indexes were all lower after treatment with Wnt agonists than those before silencing.*

***Conclusion:*** *Activating the Wnt signaling pathway can induce the increase in EPHA5 expression and enhance the stemness of non-small cell lung cancer cells.*

***Key words:*** *erythropoietin-producing hepatocellular receptor A5, non-small cell lung cancer, Wnt signaling pathway, stemness, molecular mechanism*

## Introduction

Lung cancer, a common malignancy of the respiratory system, has the highest mortality and morbidity rates in China. According to epidemiologic studies, there were about 787,000 new cases of lung cancer in China in 2019, with a morbidity rate of 57.26/100,000 and a mortality rate of 45.87/100,000, showing a trend toward younger patients [1,2]. Currently, surgical resection and systemic chemotherapy are the first-line therapeutic tools, but clinical studies have confirmed that their effect of inhibiting the recurrence and distant metastasis of lung cancer is still unsatisfactory. The specific expression of lung cancer stem cells (CSCs) is an important factor inducing clinical drug resistance, and the increased level of pluripotent transcription factors is closely related to the phenotype of CSCs [3]. Research has pointed out that some CSCs in lung cancer tissues are the root cause of tumor occurrence, invasion, metastasis, drug resistance and recurrence. Therefore, the CSC-like property is a key factor in the clinical treatment of lung cancer [4].

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In recent years, it has been discovered that erythropoietin-producing hepatocellular receptor (EPH), a subgroup of tyrosine kinases, plays an important role in embryonic development, formation and differentiation of neural vasculature, tumor invasion and metastasis [5,6]. EPH includes two subgroups, EPHA and EPHB. EPHA5 located on chromosome 4q13.1 is related to the local progression and long-term recurrence of tumors. It has been pointed out that the expression level of EPHA5 is associated with the prognosis of breast cancer patients, and it mediates trastuzumab resistance in HER2-positive breast cancer by regulating the CSC-like property [7]. Some authors have found that EPHA5 has a high expression in lung cancer tissues, but its specific mechanism of action remains unclear [8]. In the present study, therefore, the non-small cell lung cancer cell model was established through *in vitro* culture, and the molecular mechanism of EPHA5 in enhancing the stemness was explored, so as to provide a cytologic basis for therapeutic regimens able to improve the local progression and invasion of lung cancer.

## Methods

*Laboratory materials*

The following materials were used: non-small cell lung cancer NCI-H460, NCI-H1229, A549 and SPC-A1 cell lines (Chinese Academy of Sciences, Beijing, China), 0.25% trypsin, Hanks' solution and Dulbecco's Modified Eagle Medium (DMEM) (Beyotime Biotechnology, Shanghai, China), 1% streptomycin, 10% fetal bovine serum (FBS) and dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, USA), TRIzol kits, reverse transcription kits and cell counting kit-8 (CCK-8) kits (Invitrogen, Carlsbad, CA, USA), micro ribonucleic acid (miRNA/miR) rapid extraction kits and real-time polymerase chain reaction (PCR) kits (Shanghai Toscience Biotechnology Co., Ltd. , Shanghai, China), protein extraction and assay kits (QIAGEN, Hilden, Germany), rabbit anti-EPHA5 monoclonal antibodies (ABGENT, San Diego, CA, USA), stemness marker primary antibodies and horseradish peroxidase-labeled secondary antibodies (Abcam, Shanghai, China), and Wnt agonists (CHIR-99021) (Shanghai Zeye Biological Technology Co., Ltd. , Shanghai, China). The 3 kinds of EPHA5 small interfering RNAs (siRNAs) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The primer sequences are shown in Table 1.

A clean bench (BCN-1500A) was from Beijing Zhonglian Co., Ltd. (Beijing, China), an inverted optical microscope and a confocal fluorescence microscope from Zeiss (Oberkochen, Germany), a high-speed low-temperature centrifuge, a real-time fluorescence quantitative PCR amplification meter, a molecular visualizer and a microplate reader from Sigma (St. Louis, MO, USA), and an ultra-low temperature refrigerator, a horizontal shaker and a histotome from Hangzhou Rimeike Co., Ltd. (Hangzhou, China).

*Research methods*

*Cell culture and identification of EPHA5 expression*

The non-small cell lung cancer lines NCI-H460, NCI-H1229, A549 and SPC-A1 cells were cultured *in vitro*. Then, the cell suspension in the logarithmic growth phase was centrifuged at 2,000 r/min and 4°C for 10 min, and the supernatant was discarded. Later, the cell suspension was mixed evenly with 150 μL of pre-cooled radioimmunoprecipitation assay (RIPA) lysis buffer, lysed by ice bath for 5 min, and centrifuged at 8,000 r/min and 4°C for 15 min, and then the supernatant was harvested. After the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared, the sample was loaded for electrophoresis (spacer gel: 80 V and 40-50 min, separation gel: 120 V and 80 min), and transferred onto a membrane by wet process, followed by staining and sealing. Then, the sample was incubated with EPHA5 primary antibodies (1:2,000) at 4°C for 12 h, washed with tris buffered saline-tween (TBST) for 3 times, and incubated again with horseradish peroxidase-labeled secondary antibodies (1:1,500) in the dark for 1 h. After development, the protein expression level was analyzed based on the gray value of each band.

*Detection of EPHA5 silencing effect*

All EPHA5 siRNAs were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). NCI-H460 and NCI-H1229 cells with highly-expressed EPHA5 were cultured *in vitro*, prepared into cell suspension, inoculated into 6-well plates and mixed evenly with 10 μL of each of the 3 kinds of EPHA5 siRNAs. Meanwhile, a control group was set up and added dropwise with 10 μL of DMEM, and 3 replicates were set up in each group. Then, the cells were incubated in a sterile incubator



**Table 1.** EPHA5 siRNA primer sequences

| *Primer* | *Primer sequence* | |
|---|---|---|
| | *Forward (5'-3')* | *Reverse (5'-3')* |
| EPHA5 siRNA1 | CCAGUUGGAUCUCCAAUGATT | UCAUUGGAGAUCCAACUGGTT |
| EPHA5 siRNA2 | GAAGGAAAGACGUGUCAUATT | UAUGACACGUCUUUCCUUCTT |
| EPHA5 siRNA3 | GCAAUAGCUUUCCGAAAGUTT | ACUUUCGGAAAGCUAUUGCTT |

at an appropriate temperature for 6 h, and the expression of EPHA5 was detected and observed by qRT-PCR, Western blotting and immunofluorescence assay. After incubation, 50 μL of cell suspension ($1\times10^7$/mL) was aspirated in each group, from which the total RNA was isolated and purified using RNeasy kits, and reversely transcribed into complementary DNA (cDNA) using miScript II RT kit. The reaction solution was prepared in a 0.2 mL PCR tube and the reaction conditions were as follows: 40 cycles of pre-denaturation at 5°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s; the dissolution curve was analyzed at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. The expression level of EPHA5 in each group was calculated by the $2^{-\Delta\Delta Ct}$ method. Besides, the expression of EPHA5 was determined using Western blotting. In immunofluorescence assay, 2 mL of cell suspension was collected in the two groups and centrifuged at 3,000 r/min for 5 min at a low temperature, and the supernatant was discarded. Then, the cell suspension was washed twice with PBS, fixed with 4% formaldehyde for 20 min and stained according to the instructions of immunofluorescence assay kits. Finally, it was observed under a confocal fluorescence microscope.

*Detection of cluster of differentiation 133 (CD133) expression by flow cytometry*

The NCI-H460 and NCI-H1229 cell suspension was inoculated into 6-well plates, and added dropwise with 10 μL of EPHA5 siRNA3 for 6-h silencing (siRNA3 groups), with 3 replicates in each group. The EPHA5-silenced cells were cultured in a suitable environment till the logarithmic growth phase, digested with 1 mL of 0.25% trypsin and centrifuged at 1,000 r/min for 5 min at a low temperature, and the supernatant was discarded. Then, the cells were resuspended with 1 mL of pre-cooled PBS and their concentration was adjusted to $1\times10^6$/mL. Next, 100 μL of cell suspension was aspirated, added with 5 μL of PE-labeled CD133, and incubated at 4°C in the dark for 30 min. Besides, untreated cell suspension in the two groups was added with 10 μL of DMEM (control groups), with 3 replicates in each group, and the above operations were repeated. The excitation and emission wavelengths were set according to the flow cytometry operating procedures and kit instructions, and the experimental results were analyzed using FlowJo 10.5 software.

*Expression levels of sex determining region Y-box 2 (Sox2), Nanog, Kruppel-like factor 4 (KLF4) and octamer-binding transcription factor 4 (Oct4) after EPHA5 silencing*

NCI-H460 and NCI-H1229 cell suspension (5 mL) was collected and centrifuged at 2,000 r/min and 4°C for 8 min. After the supernatant was discarded, the cell suspension was washed with pre-cooled PBS, and lysed with 30 μL of RIPA lysis buffer on ice for 10 min. According to the procedure of Western blotting, the image was developed for stemness markers Sox2, Nanog, KLF4 and Oct4 (control groups). Then, an equal amount of cell suspension was harvested, treated with EPHA5 siRNA3 for 6-h silencing, and cultured in a suitable environment for 12 h, with 3 replicates in each group, and the levels of stemness markers were detected in the same way as above. Based on the gray value of each band, the relative expression level of the target protein was calculated and analyzed, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal reference. The stemness markers were quantitatively analyzed in the two groups according to the procedure of qRT-PCR.

*Detection of cell proliferative activity after Wnt agonist treatment by CCK-8 assay*

After silencing with EPHA5 siRNA3, NCI-H460 and NCI-H1229, cell suspension (5 mL) was collected, digested, washed, resuspended and inoculated in 24-well plates, followed by treatment with Wnt agonists (0 μm, 2.5 μm, 5 μm, 10 μm, 20 μm) for 6 h, with 3 replicates in each group. Then, the cells were cultured in the sterile incubator for another 12 h, and the cell proliferative activity was detected according to the CCK-8 assay operating procedure using a microplate reader. The protein expression level of β-catenin was determined by Western blotting.

*Expression levels of CD133 and stemness markers after Wnt agonist treatment*

After silencing with EPHA5 siRNA3, the cell suspension was inoculated in 24-well plates in the two groups, with 3 replicates in each group, treated with Wnt agonists (20 μm) and cultured in the incubator for 12 h. Then, the expression levels of CD133 and stemness markers Sox2, Nanog, KLF4 and Oct4 were detected by flow cytometry and Western blotting.

*Statistics*

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analyses. Measurement data were expressed as mean±SD and compared by t-test between two groups, and by one-way analysis of variance (ANOVA) among three groups. $P<0.05$ was considered statistically significant.

## Results

*Expression of EPHA5 in non-small cell lung cancer cell lines*

With GAPDH as an internal reference, the expression level of EPHA5 in non-small cell lung cancer H460 and H1229 cells was higher than that in A549 and SPC-A1 cells (Figure 1).

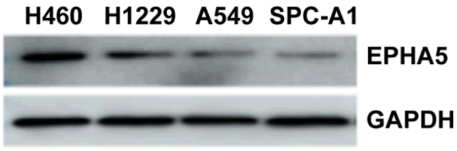


**Figure 1.** Expression of EPHA5 in non-small cell lung cancer cell lines.

*EPHA5 silencing effect*

The expression of EPHA5 in each group was detected and it was found that: 1) After silencing with siRNAs, the expression level of EPHA5 in H460 and H1229 cells declined compared with that in tumor cells and control cells (Figure 2E and 2F). 2). The expression level of EPHA5 in H460 cells was higher after silencing with siRNA2 than that after silencing with siRNA1 and siRNA3, while it had no statistically significant difference after silencing with siRNA1 and siRNA3 (Figure 2A and 2C). 3) The expression level of EPHA5 in H1229 cells was lower after silencing with siRNA3 than after silencing with siRNA1 and siRNA2 (Figure 2B and 2D).

*Expression level of CD133 in NCI-H460 and NCI-H1229 cells after silencing*

After silencing with siRNA3, the expression level of CD133 declined from 12.95% to 6.28% in H460 cells (Figure 3A), and declined from 14.47% to 5.38% in H1229 cells (Figure 3B). It can be seen that the level of non-small cell lung cancer stem cell marker protein shows a decreasing trend after EPHA5 silencing with siRNAs.

*Expression levels of Sox2, Nanog, KLF4 and Oct4 after silencing*

The results of qRT-PCR showed that the mRNA expression levels of Sox2, Nanog, KLF4 and Oct-4 in H460 and H1229 cells after EPHA5 silencing were significantly lower than those in the control group, and the differences were statistically significant ($p<0.01$, Figure 4A and 4B). The results of Western blotting revealed that with GAPDH as the internal reference, the protein expression levels of the above stemness markers in H460 and H1229 cells were down-regulated after silencing compared with those in the control group (Figure 4C and 4D).

*Cell proliferative activity*

The results of CCK-8 assay showed that the proliferative activity of H460 and H1229 cells was significantly lower after treatment with 20 μm Wnt agonists than after treatment with 0 μm



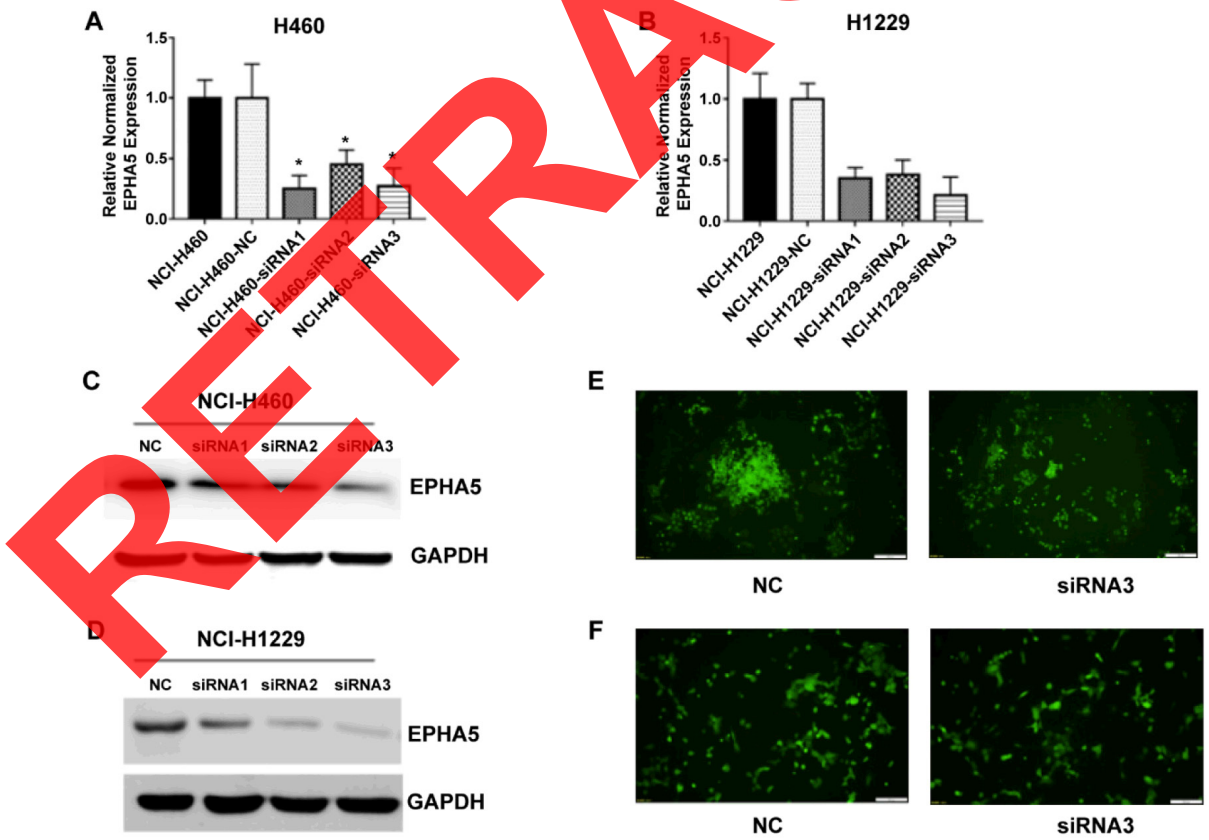


**Figure 2.** Expression of EPHA5 in non-small cell lung cancer cell lines. **A,B:** EPHA5 mRNA expression in H460 and H1229 cells in each group detected by qPCR. **C,D:** EPHA5 protein expression in H460 and H1229 cells after silencing in each group detected by Western blotting. **E,F:** It was observed under an immunofluorescence microscope that the immunofluorescence intensity of EPHA5 was lower in H460 and H1229 cells after silencing with siRNA3 than that in control group (×20). *$p<0.05$

Wnt agonists ($p<0.01$), while it declined after treatment with 10 μm Wnt agonists compared with that after treatment with 0 μm Wnt agonists ($p<0.05$). There was no statistically significant difference in the proliferative activity of H460 and H1229 cells after treatment with 2.5 μm and 5 μm Wnt agonists ($p>0.05$). Nevertheless, 5 μm Wnt agonists could better enhance the expression of β-catenin than 2.5 μm Wnt agonists, so 5 μm Wnt agonists were selected for subsequent experiments (Figure 5).

*Changes in expression levels of CD133 and stemness markers after treatment with Wnt agonists*

After treatment with Wnt agonists, the expression of CD133 in H460 and H1229 cells with EPHA5 silencing by siRNA3 was higher than that before treatment, and the expression levels of Sox2, Nanog, KLF4 and Oct4 in the above two cells were also increased compared with those before treatment. However, the expression levels of the above indexes were all lower after treatment with Wnt agonists than those before silencing. The above results demonstrate that Wnt agonists can partially reverse the inhibition of EPHA5 silencing on the expressions of stemness markers (Figure 6).

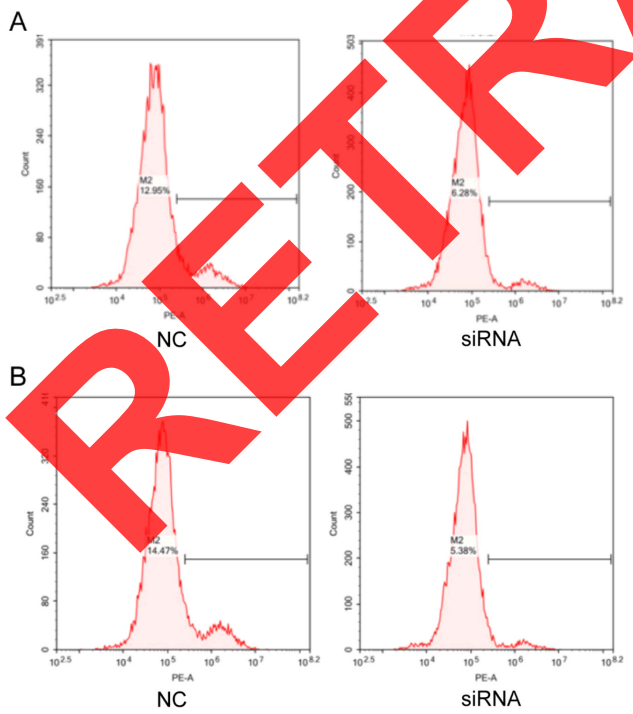


**Figure 3.** Expression of CD133 in non-small cell lung cancer cells after EPHA5 silencing with siRNA3. **A:** Flow cytometry on CD133 in H460 cells after silencing with siRNA3. **B:** Flow cytometry on CD133 in H1229 cells after silencing with siRNA3.

## Discussion

CSCs can renew themselves and produce heterogeneous tumor cells, playing an important role in the occurrence, progression, drug resistance and recurrence of lung cancer [4]. In the tumor cell model, CSCs are arranged hierarchically, forming a structure with unique abilities of self-renewal and

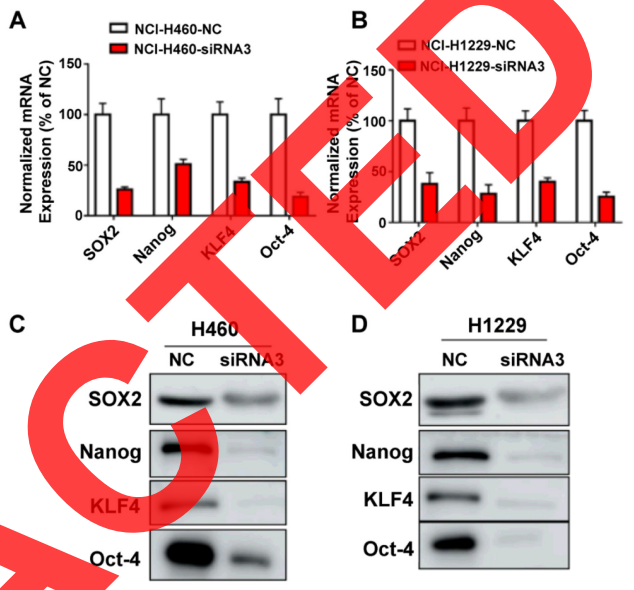


**Figure 4.** Changes in expression levels of stemness markers in H460 and H1229 cells after EPHA5 silencing. Note: **A,B:** Expression levels of stemness markers in H460 and H1229 cells after silencing with siRNA3 detected by qPCR ($p<0.01$.). **C,D:** Protein expression levels of stemness markers in H460 and H1229 cells detected by Western blotting.

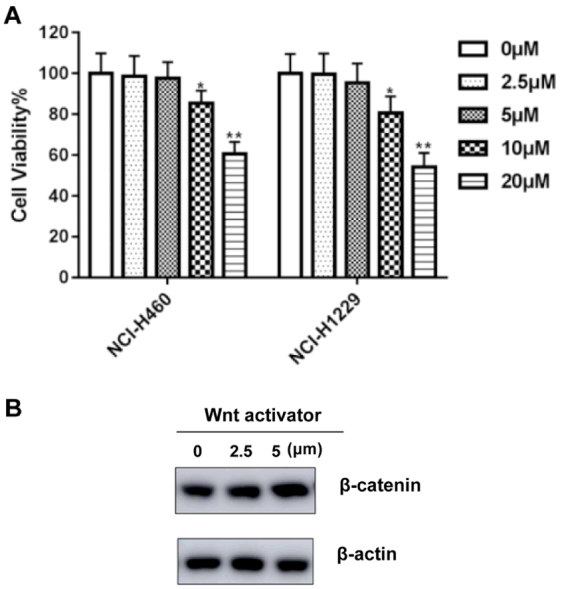


**Figure 5.** Cell proliferative activity and β-catenin expression in EPHA5-silenced cells after treatment with Wnt agonists at different concentrations. *$p<0.05$, **$p<0.01$.

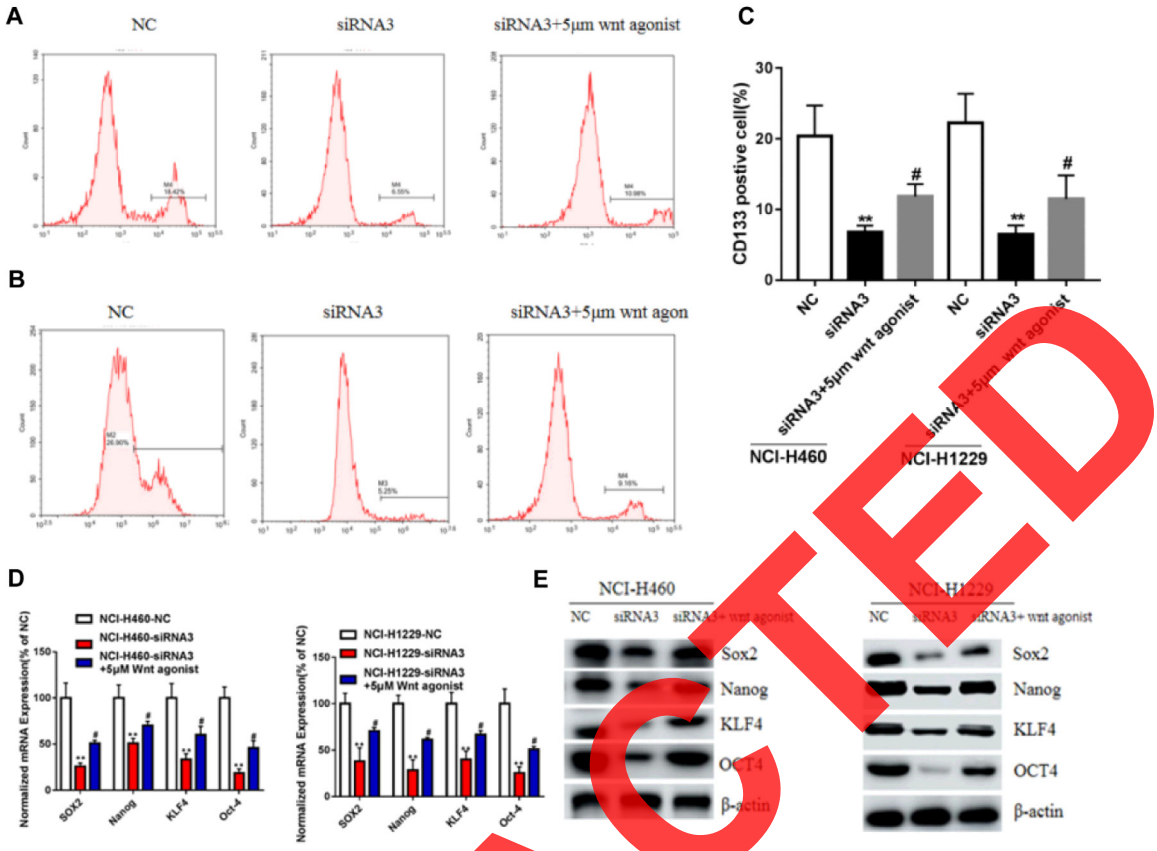


**Figure 6.** Expressions of CD133 and stemness markers in EPHA5-silenced lung cancer cells after treatment with Wnt agonists. Note: **(A, B, C)** Expression of CD133 in lung cancer cells under different treatment conditions detected by flow cytometry. **(D)** mRNA expression levels of stemness markers in lung cancer cells detected by qPCR. **(E)** Protein expression levels of stemness markers in lung cancer cells detected by Western blotting. (**p<0.01, Compared with NC group, #p<0.05, Compared with siRNA3 group).



tumor growth maintenance. Research has pointed out that CSCs keep the vitality of tumor cells through self-renewal and infinite proliferation, and their movement and migration abilities make tumor cell metastasis possible. In addition, CSCs can remain dormant for a long time, and they, with a variety of drug-resistant molecules, are not sensitive to external physical/chemical factors that can kill tumor cells, so that the risk of tumor recurrence is higher [9]. Previously, it was found that CSCs are important players in regulating the invasion and drug resistance of lung cancer, and tumor recurrence and metastasis have become the main obstacles to the improvement of the overall survival rate of lung cancer patients. However, the specific molecular mechanism of inducing stemness of lung cancer cells is currently still unclear [10]. In recent years, with the continuous deepening of research on EPH, its impact on the stemness of tumor cells has attracted extensive attention, and some studies have shown that detecting EPHA5 can effectively predict the response of non-small cell lung cancer to immunotherapy, indicating that EPHA5 may be a regulator for the activation of stemness of lung cancer cells [11].

In the present study, the non-small cell lung cancer cell lines were cultured *in vitro* and treated. The results revealed that EPHA5 had a high expression in tumor cells, and its expression in H460 and H1229 cells was higher than that in other tumor cells, suggesting that the increased expression of EPHA5 can be a marked feature of lung cancer cells. EPH genes not only participate in embryonic development, and formation and differentiation of neural vasculature, but also play an important role in tumor formation, development and metastasis. Fernanda et al [12] pointed out that EPHA5 is a regulatory molecule for the resistance of lung cancer cells to radiation, which may be involved in DNA damage repair. The radiotherapy effect on lung cancer patients with a high expression of EPHA5 is unsatisfactory, and these patients also have a worse prognosis. Zhang et al [13] found that EPHA5 mutation enhances the invasion and proliferation of non-small cell lung cancer cells and inhibits the cytotoxicity of NK cells to non-small cell

lung cancer cells. In the present study, after EPHA5 was silenced with effective siRNAs, the results of flow cytometry manifested that the expression of CD133 on the cell membrane displayed a decreasing trend. CD133 is a unique stem cell marker, and CD133-positive cells possess the abilities of long-term culture-initiating cells and long-term repopulation cells, and can induce strong cell plasticity. Huang et al [14] pointed out that the expression of CD133 is different in lung cancer cells in different grades of malignancy, and CD133-positive cells have higher stem cell activity and can grow and proliferate in the form of non-adherent tumor cells, and they also confirmed that CD133-positive cell subsets have self-renewal ability.

The results of this study showed that the expression levels of Sox2, Nanog, KLF4 and Oct4 all declined after EPHA5 silencing compared with those in the control group. Sox2, one of the important members of the Sox family, maintains the self-renewal and differentiation potential of embryonic stem cells through specific binding to the HMG domain of target genes, and plays an important role in the process of reprogramming somatic cells into pluripotent stem cells [15]. Nanog and Oct4, important stem cell transcription factors, are vital for maintaining the multi-directional differentiation potential of human embryonic stem cells and determining cell fate during early embryonic development, and they keep totipotency and self-renewal ability of stem cells mainly through forming regulatory loops, activating target genes and interacting with other signaling pathways [16,17]. Studies have demonstrated that the expression of Nanog rises in lung cancer stem cells, and reducing its expression can effectively lower the proportion of CSCs. Besides, KLF4 is a transcription factor containing three zinc-finger motifs in the carboxy terminal sequence in the KLF family, and is one of the four major factors necessary for inducing pluripotent stem cells, which can inhibit the proliferation of lung cancer cells through suppressing the level of placenta-specific protein 8 [18]. Combined with the results in this study, it is speculated that EPHA5 silencing may affect the proliferation and differentiation of tumor cells *via* reducing the protein expression levels of stemness markers.

In addition, after treatment with Wnt agonists at an effective concentration, the expression of CD133 in EPHA5-silenced lung cancer cell lines was significantly up-regulated compared with that before treatment, and the expression levels of Sox2, Nanog, KLF4 and Oct4 also rose compared with those before treatment. As it has been pointed out in studies, Wnt signals are involved in cell proliferation, differentiation, migration and apoptosis, and have correlations with the occurrence and development of a variety of tumors. When the Wnt pathway is activated, Wnt protein can result in the accumulation of dephosphorylated β-catenin in the cytoplasm, and then β-catenin enters the nucleus and interacts with T cell factors/lymphoid enhancer factors, thereby activating the transcription of downstream target genes [19,20]. To sum up, the expression levels of the above indexes after treatment with Wnt agonists were still lower than those in the control group, but Wnt agonists could partially reverse the inhibition of EPHA5 silencing on the expressions of stemness markers. It can be seen that EPHA5 silencing can weaken the stemness of lung cancer cells, which may be achieved by inhibiting the Wnt signaling pathway.

## Conclusions

In conclusion, the results in this study demonstrated that EPHA5 is an important regulatory factor for the stemness of lung cancer cells, and activating the Wnt signaling pathway can induce the stemness. Such findings provide a cytologic basis for the inducing mechanism of lung cancer cell proliferation, invasion and drug resistance.

## Conflict of interests

The authors declare no conflict of interests.



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