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Reviewer A

The work aimed to assess the effect of BPHL knockdown on the proliferation, apoptosis, colony formation, metastasis, and cell cycle of cancer cells. Also, explored the effect of BPHL knockdown on tumor growth in a mouse model of tumor implantation in nude mice.
There are some minor revisions:
1-First, The article has some typo mistakes, that must be corrected. e.g. in line 30 “Biphenyl hydrolase-like (BPHL) the type of font should be correct.
Also, in line 153, replace ul with µL.
In line 166, implantation should be started with a capital letter.

Reply 1: Thank you very much for the reviewer's comments, I have corrected the typography and typos in the article.
Changes in the text: see line 30“Biphenyl hydrolase-like (BPHL) ”; see line 165“µl”; see line 183 “Implantation”.

2-# Abstract, in Methods should be more details, in addition, The conclusion contains repetitive sentences and must be rephrased again. “In this study, the data indicate that BPHL potentially promotes proliferation, inhibits apoptosis, and increases colony formation and metastasis in lung cancer. Overall, our study suggests that BPHL potentially contributes to the carcinogenesis, growth, impaired apoptosis, increased colony formation, and metastasis of lung cancer”.

Reply 2: The reviewer's comments are greatly appreciated, and the Methods section of the Abstract has been revised as requested. I've optimized the conclusion section by removing repeated statements.
Changes in the text: see line 62-70 " BPHL knockdown NCI-H1299 and A549 cells demonstrated decreased proliferation, as measured by Celigo cell counting. The MTT assay results were consistent with Celigo cell counting. Caspase 3/7 activity increased significantly in the NCI-H1299 and A549 cells after shBPHL knockdown. Decreased colony formation in the NCI-H1299 and A54 cells after shBPHL knockdown, as measured by crystal violet staining. Transmigration assay using a Transwell demonstrated that there were significantly fewer migrating cells in the lower chamber in the BPHL knockdown NCI-H1299 and A549 cells. Cell cycle analysis by PI staining and FACS." Line 83-84"Overall, our study suggests that BPHL may be a gene that promotes tumor growth in lung cancer."

3-#Methods: the method lacks chemicals and kits used in this study, and also lacks references in all techniques and experiments such as MTT assay, Cell apoptosis, Colony formation, Cell migration, The cell cycle …..etc. so the method needs more details.

Reply 3: I have added details to the experimental methods section and supplemented the contents of the kits and references.

Changes in the text: see line 153-182 "The cells were counted with an MTT （Genview,Cat.N0.JT343）assay and Celigo (Nexcelom) using a 96-well plate (100 µL/well). A total of 200 cells were seeded in each well and incubated for 120 hours at 37℃ in a humidified atmosphere with 5% CO2.(14, 15) Images were taken every 24 hours to evaluate the effect of BPHL knockdown on cell proliferation.

Cell apoptosis, as measured by caspase 3/7(Promege,Cat.N0.JG8091) activity, was tested according to the manufacturer's recommended protocol. Briefly, 10,000 cells in 100 µL were seeded into a 96-well plate and incubated for 36 hours at 37℃ in a humidified atmosphere with 5% CO2. The caspase 3/7 activity was then assayed.(16)

Colony formation was evaluated in a six-well tissue culture plate. A total of 800 cells were seeded in 2 mL of culture media and were cultured for 5 days at 37℃ in a humidified atmosphere with 5% CO2. On day 5, 1,000 µl of crystal violet(Sangon Biotech,Cat.N0.CB0331) was added to stain cells for 20 min, followed by imaging and colony counting. The number of colonies containing≥50 cells was counted using a microscope. (17, 18)The experiment was performed with three replicates for each cell line.

Cell migration toward the serum was performed in a Transwell chamber(Corning,Cat.N0.3422) to investigate the role of BPHL on cell migration. A 24-well plate with an upper chamber volume of 100 µL and a lower chamber volume of 600 µL was used (corning). A total of 100,000 cells were seeded into the upper chamber and incubated for 20 hours, followed by counting cells in the lower chamber.For quantification, the cells in the lower compartment were stained with Giemsa stain(Sigma,Cat.N0.32884) and counted in five randomly chosen fields (×200) under a microscope. (19)The experiment was performed with three replicates.

The cell cycle was examined by PI staining(Sigma,Cat.N0.P4170) of the DNA in cells followed by measurement of the PI signal by Fluorescence activated cell sorter (FACS)(Millipore, Billerica, MA, USA). The cells were seeded into a six-well plate (2 mL/well) and cultured for 4 days to reach 80% confluence.(20)"

4-#Results: Figure 1 should be more clear and high resolution. And the results need some explanations.

Reply 4: Figure 1 I have modified as requested. The results of Figure 1 and Table 1 were also analyzed.

Changes in the text: see line 436 "Figure 1" and see line 210-216 "the expression difference in BPHL genes between The cancer and adjacent tissues in TCGA database (data obtained from both The tumor and adjacent normal tissues of 57cases as well as The pathology data in The Lung adenocarcinoma (Luad) category) was demonstrated by The FC (2.253) and p value (8.87E-27) . Based on bioinformatics data, we performed a preliminary experiment and found that expression difference in BPHL genes between the cancer and adjacent tissues in the lung adenocarcinoma."

5-# Discussion: The discussion is very brief and should be written in detail better than that.

Reply 5: In the discussion section, we further analyze and summarize the experimental results, and analyze the shortcomings of this experiment. The research group also conducted further discussions on the future research direction.

Changes in the text: see line 281-327 "This illustrates that inhibition of BPHL expression can reduce the malignancy of tumor cells, including reduced proliferation, increased apoptosis, reduced colony formation, and reduced metastasis, and by further experiments, it was found that most of the tumor cells that inhibited BPHL expression stopped in the prophase of mitosis, further confirming that inhibiting BPHL expression would reduce the malignant degree of tumor cells, it causes a decline in pathogenicity and may accelerate death. Furthermore, The TABLE1 data showed that The difference of BPHL gene expression between The tumor and The adjacent tissue was significant in The current and future lung adenocarcinoma tumor database, namely that BPHL knockdown tumor cells grew more slowly and weighed less in vivo. These data suggest BPHL potentially promotes proliferation, inhibits apoptosis, and increases colony formation and metastasis in lung cancer.

According to current statistics, there were 19.29 million new cancer cases and 9.96 million cancer deaths in 2020, including 2.26 million new cases of breast cancer and 2.2 million new cases of lung cancer, however, lung cancer is still the world's leading cause of death, accounting for 1.8 million deaths, or 22.7% of the total, far exceeding other cancer types. China has 4.57 million new cases of cancer, accounting for 23.7% of the global total, and 3 million cancer deaths in China, accounting for 30% of the global total. Lung cancer is one of the malignant tumors with the highest morbidity and mortality in China, with 820,000 new cases of lung cancer in 2020, accounting for 18.0% of the total, leading by far the number of lung cancer deaths, reaching 710,000, it accounts for 23.8% of all cancer deaths in China. Current data show that the overall incidence of lung cancer is on the rise worldwide (25). With the rapid development of medicine, the treatment of lung cancer has also undergone tremendous changes, from the traditional thoracotomy, later, micro-invasive surgery (26-29) , systemic chemotherapy, molecular targeted therapy(30) , immunotherapy, radiotherapy and so on were gradually developed, but the long-term effect of patients with advanced lung cancer was still not ideal. At present, it is thought that the occurrence of lung cancer may be related to long-term smoking, environment and genetic factors, but the specific mechanism of the occurrence and development of lung cancer is not clear. Therefore, we should go deep into the basic research of the occurrence and development of lung cancer, especially lung adenocarcinoma, fully understand the molecular mechanism of proliferation and metastasis, and search for effective and specific key regulatory genes for the occurrence and development of lung cancer, it is of great value not only in predicting the prognosis of lung adenocarcinoma, but also in improving the therapeutic effect and reducing the mortality of lung adenocarcinoma.

At present, there are no studies on its role in tumor tissues and the mechanism underlying its function is unknown. Our study found that BPHL promoted lung cancer carcinogenesis, which warrants further clinical and mechanistic studies. But this study only completed transfection of shRNA to suppress the expression of the BPHL gene; In order to better understand the mechanism of BPHL-induced carcinogenesis, we will further study the overexpression of BPHL gene in lung adenocarcinoma cell lines, and clarify the function of BPHL oncogene and the cellular pathway of BPHL-induced carcinogenesis, further molecular studies are needed to

identify the genetic basis of BPHL target proteins and aberrant expression of BPHL in NSCLC for their possible subsequent development of targeted genes for this gene. "

6- #Finally, the number of references is just 18 references, some references should be added.

Reply 6: Based on your comments, we have added the experimental methods, discussion section, including some references.
Changes in the text: see line 348-415 "References

1. Li Y, Chao Y, Fang Y, et al.: MTA1 promotes the invasion and migration of non-small cell lung cancer cells by downregulating miR-125b. 2013:32-3.
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Reviewer B

In the study by Ren et al., the role of BPHL (Biphenyl hydrolase like protein) was investigated in non-small cell lung cancer cells (NSCLC). They found that siRNA knockdown led to decreased proliferation, migration/invasion, and enhanced apoptotic levels. In nude mice test, they have observed a reduced tumor growth in vivo. They made the conclusion that BPHL might be a tumor suppressor in NSCLC.

The topic is interesting, however, the study is methodically not well performed. All the data are merely based on the siRNA knockdown. Given the fact that downregulated gene/protein expression by siRNA can only be maintained up to 6 days, how the siRNA constructs could be used for a relatively long-term in vivo nude mice test with the observation time more than 4 weeks? Besides, only a handful of methods, mainly cell-based functional assays, were applied, and no obvious novelty is present in the study.

It would be more convincing if the data present are based on stable shRNA transfection or overexpression models.

Comment 1: Given the fact that downregulated gene/protein expression by siRNA can only be maintained up to 6 days, how the siRNA constructs could be used for a relatively long-term in vivo nude mice test with the observation time more than 4 weeks?

Reply 1:In the text of Lentivector expressing Ribo Nucleic Acid Interference (RNAi) for BPHL (Line132:hich was shuttled into the GV115 lentivector, followed by virus packaging.), Lentivector-expressing RNAi for BPHL and screening human lung carcinoma cell lines that express B PHL (Line212 : NCI-H1299 and A549 were transduced with GV115-RNAi-BPHL lentivirus and a control lentivirus.), Figure 2, mentioned that the designed siRNA was

integrated into the lentivirus, transfected cell line, which is continuous interference through shRNA transfection The expression of BPHL has good stability, and it can inhibit the expression of BPHL for several weeks. It will be more accurate to use shRNA in the Highlight box and the abstract. Thanks to the reviewers for their valuable comments.
Changes in the text: see line32"short hairpin RNA(shRNA)",see line 73"short hairpin RNA(shRNA)"

Comment 2: Besides, only a handful of methods, mainly cell-based functional assays, were applied, and no obvious novelty is present in the study.It would be more convincing if the data present are based on stable shRNA transfection or overexpression models.
Reply 2: Thanks to the reviewers for their valuable comments. This is the limitation of our study. Our experiment only inhibited the expression of BPHL by shRNA. In the revised Discussion section, we also acknowledged the limitations of the experiment. In the follow-up experiments, we will Through shRNA overexpression of BPHL, the function of BPHL is further verified, and further in-depth research is carried out on the signaling pathway.
Changes in the text: see line320-327“But this study only completed transfection of shRNA to suppress the expression of the BPHL gene; In order to better understand the mechanism of BPHL-induced carcinogenesis, we will further study the overexpression of BPHL gene in lung adenocarcinoma cell lines, and clarify the function of BPHL oncogene and the cellular pathway of BPHL-induced carcinogenesis, further molecular studies are needed to identify the genetic basis of BPHL target proteins and aberrant expression of BPHL in NSCLC for their possible subsequent development of targeted genes for this gene.”.

Reviewer C

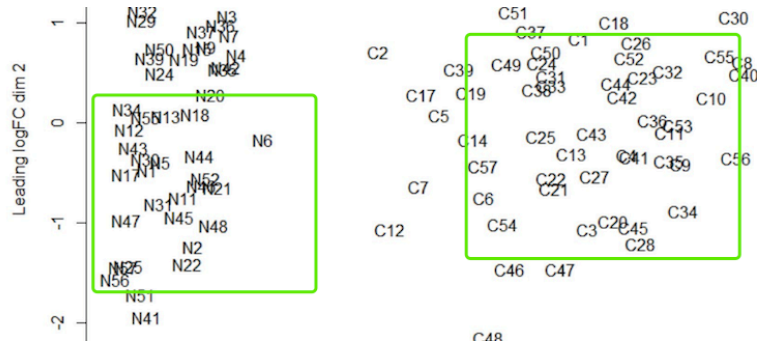
1. Please check if the citations of references need to be added in the below sentence since you mentioned the previous “studies”.

91 **#Introduction**
92 According to previous studies, the Metastasis-associated gene 1 (MAT1) protein, an
93 important regulator for tumor metastasis, promotes tumor metastasis in Nonsmall-cell
94 lung cancer (NSCLC). The results from our earlier study demonstrated that small

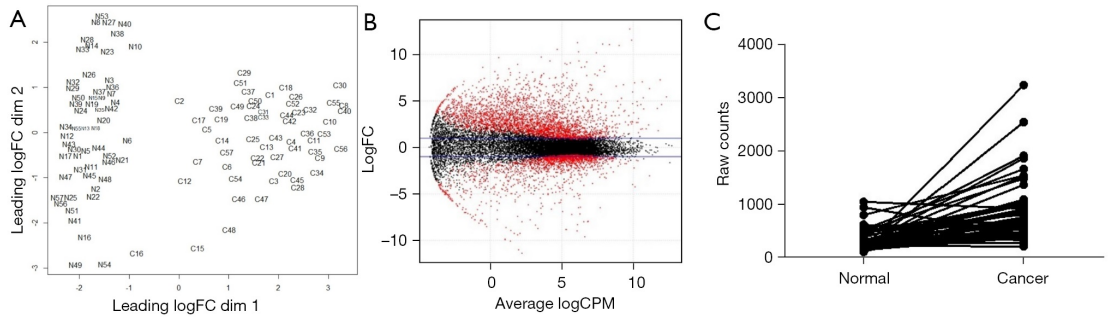
Reply: The text in this paragraph is describing this "studies", and the relevant literature reference (1) is at the end of this paragraph.
Changes in the text: see line 95“.(1)”

2. Table 1-2:
The titles for Table 1-2 are the same one. It’s not allowed.
Reply: I modified the titles of 2 tables.
Changes in the text: see line 436“Table 1 BPHL gene-related information” and see line 444“Table 2 Expression of BPHL gene in TCGA database”.

3. Figure 1:
1) The gene names are overlapped and not clear. Please modify.

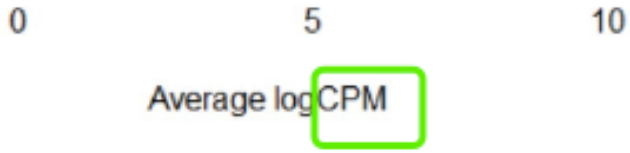


Reply: I have modified the Figure1A as requested.
Changes in the text: see line 453.



2) Please indicate the meaning of colorful dots in Figure 1 B legend.
Reply: I have added annotations as requested.
Changes in the text: see line 460-462“Genes with a statistical test P value less than 0.05 are considered as differentially expressed genes that meet the null hypothesis (marked in red in the figure)”.

3) Please indicate the full name of “BPHL” and “CPM” in the legend.



Reply: I have added the full name as requested.
Changes in the text: see line 466-467“CPM, Counts Per Million; BPHL, biphenyl hydrolase-like;”.

4. Figure 2:
1) Please indicate the meaning of ** and the full name of “BPHL” in the legend.
Reply: I have added notes and full name as requested.
Changes in the text: see line 481-482“**, P < 0.01 compared with shCtrl and shBPHL lentivirus treatment group.” and see line 479“BPHL, biphenyl hydrolase-like”.

2) The below two parts have the same meanings. It’s unnecessary to repeat.

Chain Reaction (qPCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene, and the 5' and 3' primers were CCATTTCAGCACCTCGGTA and CAGCATCTTTTGCATCCCT, respectively, with an amplicon size of 266 bp (Figure 2A). NCI-H1299 and A549 cells expressing the highest and lowest BPHL Messenger Ribonucleic Acid (mRNA), respectively, were chosen for the subsequent experiments.

NCI-H1299 and A549 cells were cultured in RPMI 1640 + 10% FBS and transduced with the GV115-RNAi-BPHL and control lentiviruses. At 72 hours post-transduction, the cells displayed good viability. The transduction efficiency reached 80%, as measured by the percentage of fluorescence-positive cells (Figure 2B). The mRNA levels were measured by qPCR to confirm the efficiency of the knockdown (Figure 2C). Western blot demonstrated that there was a significant decrease in BPHL at the protein level, as compared to the controls (Figure 2D).

##Lentivector-expressing RNAi for BPHL and screening human lung carcinoma cell lines that express BPHL

Three common human lung carcinoma cell lines, A549, NCI-H1975, and NCI-H1299, were chosen to test for their gene expression of BPHL using qPCR. GAPDH was used as a reference gene, and the 5' and 3' primers were CCATTTCAGCACCTCGGTA and CAGCATCTTTTGCATCCCT, respectively, with an amplicon size of 266 bp (Figure 2A). NCI-H1299 and A549 cells expressing the highest and lowest BPHL mRNA, respectively, were selected for the subsequent experiments.

NCI-H1299 and A549 were transduced with GV115-RNAi-BPHL lentivirus and a control lentivirus. At 72 hours post-transduction, the cells displayed good viability. The transduction efficiency had reached 80%, as measured by the percentage of fluorescence-positive cells (Figure 2B). mRNA levels were measured by qPCR to confirm the efficiency of the knockdown (Figure 2C). Western blot demonstrated that there was a significant decrease in BPHL at the protein level, as compared to the controls (Figure 2D).

Reply: I have edited the duplicate content as requested.

Changes in the text: see line 139-148"Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene, and the 5' and 3' primers were CCATTTCAGCACCTCGGTA and CAGCATCTTTTGCATCCCT, respectively, with an amplicon size of 266 bp . The screened lung cancer cell line was used as a follow-up experimental cell line.

NCI-H1299 and A549 cells were cultured in RPMI 1640 + 10% FBS and transduced with the GV115-RNAi-BPHL and control lentiviruses. At 72 hours post-transduction, the cells displayed good viability.Detect the transfection effect of cells as measured by the percentage of fluorescence-positive cells. Finally, the expression of mRNA and protein was detected by qPCR and Western blot."and see line 220-229"GAPDH was used as a reference gene, NCI-H1299 and A549 cells expressing the highest and lowest BPHL mRNA, respectively, were selected for the subsequent experiments.

NCI-H1299 and A549 were transduced with GV115-RNAi-BPHL lentivirus and a control lentivirus. At 72 hours post-transduction, the cells displayed good viability. The transduction efficiency had reached 80%, as measured by the percentage of fluorescence-positive cells (Figure 2B). mRNA levels were measured by qPCR to confirm the efficiency of the knockdown (Figure 2C). Western blot demonstrated that there was a significant decrease in BPHL at the protein level, as compared to the controls (Figure 2D)."

5. Figure 3:

1) Please indicate the staining method and magnification in Figure 3A-B and Figure 3F legends.

Reply: The Celigo cell counting experiment in Figure 3A-B uses fluorescence imaging without the use of dyes. This experiment was photographed by the Celigo instrument and no microscope was used. The staining method and magnification have been noted in the Figure 3F legend.

Changes in the text: see line 502“(Giemsa stain, ×200) ”.

2) Please indicate the staining method in Figure 3E legend.

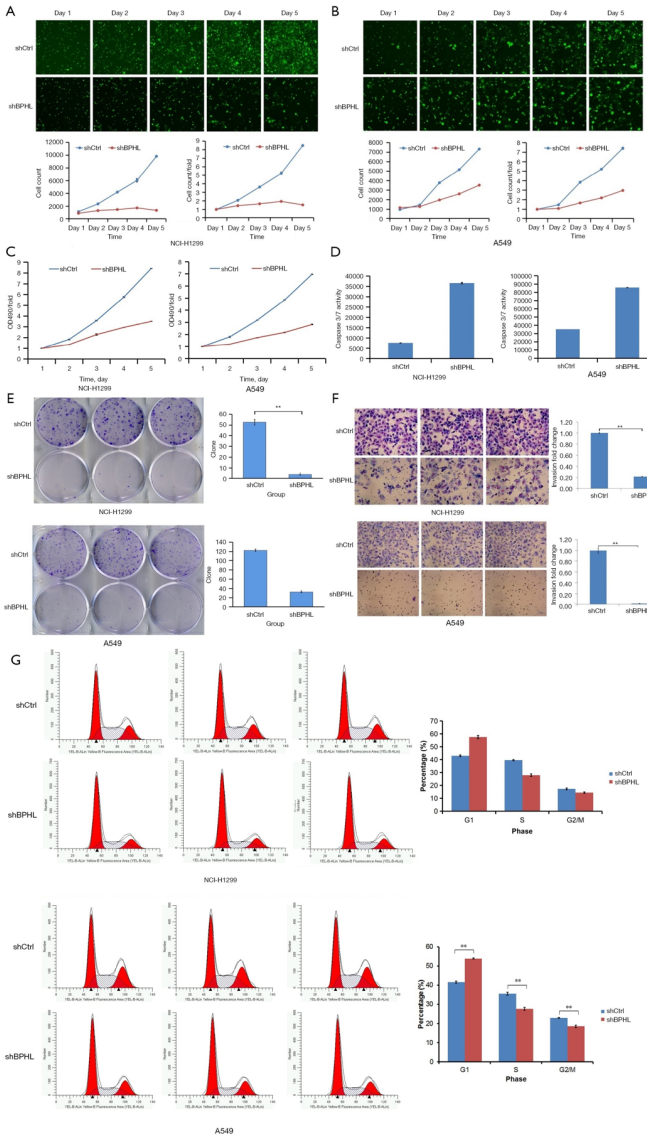
Reply: The staining method have been noted in the Figure 3E legend.

Changes in the text: see line 500“crystal violet staining.”

3) Figure 3G is too vague; please resubmit your Figure 3 in higher resolution.

Reply: I have modified the Figure3G as requested.

Changes in the text: see line 491.



4) Please indicate the meaning of ** and the full name of “BPHL”, “PI”, “FACS”, “OD” in the legend.

Reply: I have added notes and full name as requested.

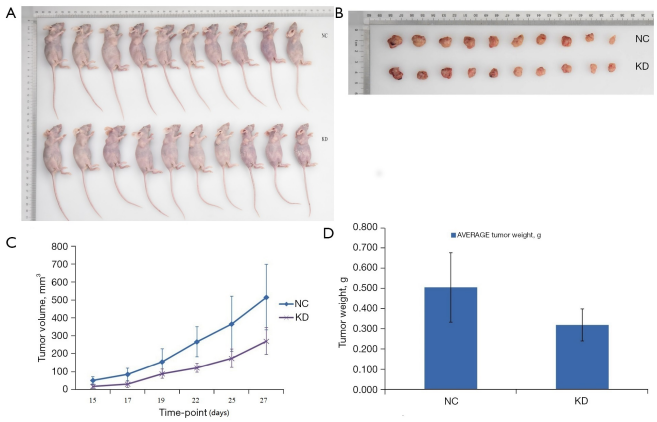
Changes in the text: see line 503-505 “**, $P < 0.01$ compared with shCtrl and shBPHL lentivirus treatment group; BPHL, biphenyl hydrolase-like; PI, Propidium Iodide; FACS,Fluorescence activated cell sorter; OD, optical density.”

5. Figure 4:

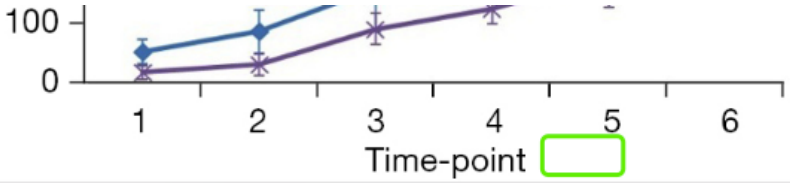
1) The scale bars in rules of Figure 4A are too vague; please resubmit your Figure 4 in higher resolution.

Reply: I have modified the Figure 4A as requested.

Changes in the text: see line 507.



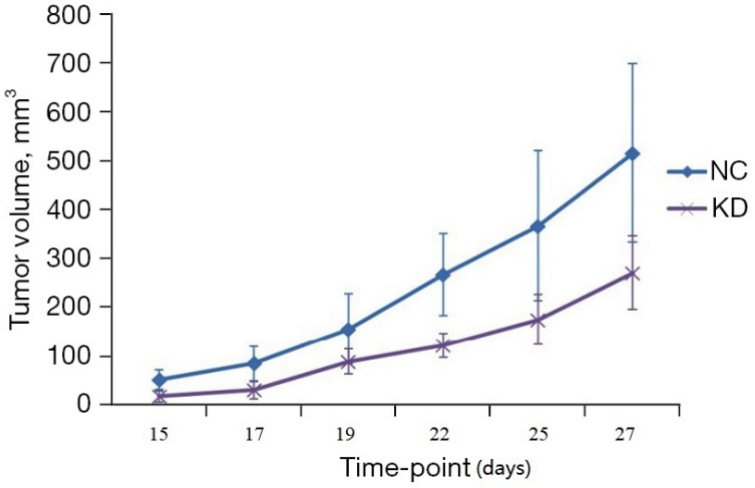
2) Please add unit for Time in the x-axis of Figure 4C.



Reply: I have modified the Figure 4C as requested.

Changes in the text: see line 507.

C


800
700
600
500
400
300
200
100
0
Tumor volume, mm3
15
17
19
22
25
27
Time-point (days)
NC
KD