Answering Reviewers Letter

Dear editors and reviewers:

Thank you very much for your letter and the comments on our paper entitled "**DARPP-32 promotes colorectal cancer growth by activating the PI3K/AKT pathway**". We have checked the manuscript and revised it according to the comments and have carefully revised our paper based on the comments of reviewers. The point-to-point responses to the reviewers′ comments are presented below.

We also appreciate our dear reviewers for giving us precious advices,

which are important for us to improve the quality of our work.

Reviewer 1# (04049611)

It is my great honor to receive your comments about our paper, and responses are presented below. Thank you very much!

1. Previous study demonstrated that PP-1 directly dephosphorylates AKT to modulate its activation (Cell Death & Differentiation. 2010, 17(9): 1448-62). Moreover, the authors described that DARPP-32 would act as a PP-1 inhibitor when the Thr34 residue of DARPP-32 is phosphorylated by PKA. Please conduct some experiments to check the phosphorylation level of DARPP-32 at Thr34 residue and the status of the PP-1 activity in CRC cells with DARPP-32 overexpression.

Thank you for your comment. Previous study demonstrated that dual effects of phosphorylation of DARPP-32 in regulation of PKA and PP1 activities. Dopamine D1 receptor activation of PKA and phosphorylation of Thr 34 by PKA converts DARPP-32 into an inhibitor of PP1. The activation of PKA and inhibition of PP1 synergistically increase phosphorylation of various substrates3.Conversely, phosphorylation of DARPP-32 at Thr 75 by Cdk5 causes inhibition of PKA and activation of PP1, and synergistically reduces phosphorylation of various substrates (Nature. 1999 Dec 9;402(6762):669-71). "DARPP-32 and t-Darpp protein products of PPP1R1B: old dogs with new tricks" is a paper that further summarizes and describes the role of DARPP-32. This study represents an initial exploration of the role of DARPP-32 in colorectal cancer. The specific mechanisms of action and related proteins, such as PP1, still need to be further elucidated in future experiments.

2. The authors implied that overexpression of DARPP-32 could promote cell proliferation, migration, and invasion of CRCs, whose phenomena may be induced by DARPP-32-mediated enhancement in the activation of the PI3K/AKT pathway. For clarifying the significance of PI3K/AKT activation in DARPP-32-mediated several phenomena in CRC, PI3K or AKT blockers should be applied in the present study.

Thank you for your comment. To elucidate the significance of

PI3K/AKT activation in the various phenomena mediated by DARPP-32 in CRC, we performed additional experiments where we applied an AKT inhibitor and examined the expression of phosphorylated AKT (P-AKT). The results are shown in Supplementary Figure 2.

3. GAPDH expression was significantly up-regulated in human colorectal carcinoma tissues (J Bioenerg Biomembr. 2012, 44(1):117-25), and over-expression of β-tubulin is associated with poorer outcomes in colorectal cancer (Cells. 2019, 8(1):25). These articles implied that the expression levels of GAPDH and β-tubulin may be altered in CRC, and thus these molecules seem inappropriate to be used as the internal controls in the present study.

Thank you for bringing up this point. In this study, the use of GAPDH and β-tubulin as internal controls has certain limitations. However, it is important to note that most of the comparisons were conducted within the same cell line, and the interventions were specifically targeted at the protein of interest, DARPP-32. No intentional manipulation of the expression of the reference genes was performed. Additionally, it is known that the expression of reference genes can vary in abundance across different cell lines. Nevertheless, GAPDH(J Exp Clin Cancer Res. 2021 Sep 28;40(1):304) and β-tubulin(J Exp Clin Cancer Res. 2020 Jul 22;39(1):141)have been widely used as reference genes in previous studies. Therefore, considering these factors, we have

chosen to use GAPDH and β-tubulin as internal controls.

4. The authors should explain the rationale why SW480 rather than other CRC cells with higher DARPP-32 were chosen for the RNA-seq study. Can SW480 cells be a typical representative of CRC?

Thank you for your comment. This study includes the use of RNA-seq to study the effect of DARPP-32 changes on downstream signaling pathways, and to explore the regulatory mechanism of DARPP-32. SW480 cells are widely used and well-established colorectal cancer cell lines that have been extensively studied in various research areas. While SW480 cells may not represent all subtypes or characteristics of colorectal cancer, they can still provide valuable insights into certain aspects of the disease. The sequencing results provided us with insights for further experiments, and it is necessary to validate these findings based on the results. Taking all these considerations into account, we chose the SW480 cell line for RNA-seq.

5. Cancer cells must have some genetic mutations and altered signaling transduction, and these changes may disturb our observation of the intracellular roles of DARPP-32. The experiments regarding the over-expression of DARPP-32 should be re-conducted in normal cells, such as NCM460, rather than CRC cells if the experimental purpose is to understand the carcinogenic possibility of over-expressed DARPP-32 in normal cells.

Thank you for raising this important point. Our experimental goal was to understand the impact of DARPP-32 on proliferation, migration, and other functions in colorectal cancer cell lines. Therefore, we chose to overexpress DARPP-32 in colorectal cancer cell lines HCT116 and SW480, which exhibit relatively low expression levels. Conversely, we knocked down DARPP-32 expression in HT29 and LOVO cell lines, which have relatively high expression levels of DARPP-32. By doing so, we aimed to observe any changes in the functions of colorectal cancer cells. We appreciate your suggestion and agree that future studies could include experiments in normal cells to provide further insights into the potential effects of overexpressed DARPP-32 in non-cancerous settings.

6. In Figure 1G. Please check the correctness of the data or the label of the y-axis because in general the relative expression level of the control group should be defined as 1.

Thank you for pointing out the concern regarding Figure 1G. In general, the relative expression level of the control group is often defined as 1 for comparison purposes. In Figure 1G, the y-axis represents the mRNA expression levels of DARPP-32 in different cell lines. The control group is represented by the NCM460 cell line. However, we did not normalize the expression levels of the control group to a value of 1. Instead, we directly compared the mRNA expression levels. As a result, the control group was not defined as 1.

7. In Figures 2 and 4. The cytotoxic effect induced by silencing DARPP-32 should also be evaluated in normal cells (NCM460) to understand whether the drug targeted on DARPP-32 will cause significant cytotoxicity in normal cells.

Thank you for your suggestion. Evaluating the cytotoxic effect induced by silencing DARPP-32 in normal cells (NCM460) is indeed crucial to assess the potential cytotoxicity of the drug targeted at DARPP-32 in normal cellular contexts. However, in this study, our focus was primarily on investigating the impact of DARPP-32 on colorectal tumor cell function, and therefore we did not assess its toxicity in normal cells. We agree that including the evaluation of normal cells in future studies would provide valuable insights into the specificity and safety of targeting DARPP-32 as a therapeutic approach.

Minor Comments: 1. Please check the contextual correlation of the sentence "Epidermal growth factor receptor (EGFR) mutant in non-small cell lung cancer" (Lines 105-106).

I apologize for the misunderstanding. The correct translation of the sentence "DARPP-32 isoforms are overexpressed to promote the "bypass signaling" of epidermal growth factor receptor (EGFR) in non-small cell lung cancer". Thank you for your comment.

2. The experimental results or conclusions of the present study should not be described in the Introduction section.

Thank you for your comment. The suggested changes have been made to the revised manuscript.

3. Please provide the clinical demographics of the tissue donors and the approval number of the IRB study.

The clinical demographics of tissue donors are provided in the attached document "Clinical Demographic Data". The IRB approval number for this study is No. 2020-358. Thank you!

4. Please carefully check typing or grammatical errors as well as confirm the correctness of the style (uppercase or lowercase) of the words or terms. For example, “CO2”, “10%SDS-PAGE.After”, “We thank Professors …”, “molecular pharmacological in …”, “… approved this animal experiments”

Thank you for your comment. I apologize for any errors or inconsistencies in the manuscript. The suggested changes have been made to the revised manuscript.

5. Abbreviations should be defined at the first mention and then present consistently. For example, TAM, siDARPP-32, “KI-67 vs. Ki-67”, OE

Thank you for your comment. The suggested changes have been made to the revised manuscript (Line 129 274 281 515).

6. Please provide the mRNA accession number for the genes detected in the qPCR assay.

Thank you for your suggestion. The mRNA accession number for the gene detected in the qPCR assay is NM_032192.4. Thank you!

7. Please provide the sequences of siRNA2 and siRNA3 as well as the working concentration of all siRNA. Is there a negative control siRNA applied?

The sequence of siRNA2 (Line 162) is as follows: 5′-GAUAG UACUAGCAAGUAUACU-3′(sense) and 5′-UAUACUUGCUAGUAC UAUCUU-3′ (antisense). The sequence of siRNA3 (Line 164) is a s follows: 5′-AGAUAUGUAUCUUAUAUAAAC-3′ (sense) and 5′-U UAUAUAAGAUACAUAUCUUG-3′ (antisense). The working concen tration of siRNA is 20 nM. We used a negative control siRNA in our experiment. Thank you!

8. Please leave a blank space between the value and its unit. For example, "100ul 50μM" (Line 171). Besides, the volume unit should be corrected as "μl" rather than "ul".

Thank you for your comment. The suggested changes have been made to the revised manuscript (Line 170).

9. Please provide detailed information (e.g. catalog number, company, city, and country) of commercial kits and antibodies.

Thank you for your comment. The suggested changes have been made to the revised manuscript (Line 217-220).

10. Please describe the procedure of data conversion of

immunoblotting and qPCR assays in the Materials and Methods section.

Thank you for your comment. The suggested changes have been made to the revised manuscript.

11. Please provide sequencing depth in the RNA-seq experiment. Besides, it's a wrong description that the quality and integrity of total RNA product were determined by using a NanoDrop spectrophotometer (Lines 204-205).

I apologize for using incorrect information. The revised manuscript now accurately states that the concentration of the product was determined with the aid of a NanoDrop spectrophotometer. Thank you!

12. Please provide the gender of BALB/c naked mice (Line 230).

Thank you for your comment. The suggested changes have been made to the revised manuscript (Line 228).

13. "The student's test" is an incorrect name (Line 240). Besides, other statistical analyses should be described in the Materials and Methods section.

I apologize for the incorrect name. The correct name for the statistical test is "Student's t-test." Thank you for pointing out the mistake. Other statistical analyses have been provided in the Materials and Methods section to address this concern (Line 238). Thank you!

14. Please define what is "normal CRC samples" (Lines 247-248).

I apologize for the mistake. The correct description should be "adjacent normal tissue" or "normal adjacent tissue" rather than "normal CRC samples." These refer to the tissue samples collected from the non-cancerous region adjacent to the tumor site in patients with colorectal cancer. These adjacent normal tissues are used as controls to compare with the cancerous tissues and provide a reference for studying the molecular or pathological changes specific to colorectal cancer. Thank you!

15. Some references miss information regarding volume, issue, page number, or article number.

Thank you for your comment. The suggested changes have been made to the revised manuscript.

16. In Figure 1. What do the red and gray bars mean? What do the abbreviations, COAD and READ, mean? Is there any substantive difference between Figure 1A and 1B for the purpose of the experiment? It's a redundant description "**P<0.0001" (Line 503).**

In our study, the red and gray bars in the results represent scale bars used for size reference in the images. In Figure 1, the red scale bars indicate a length of 200 μm, while the black scale bars indicate a length of 50 μm. In our study, the abbreviations "COAD" and "READ" refer to specific subtypes of colorectal cancer. COAD stands for "Colon Adenocarcinoma," which represents cancer that originates in the colon.

READ stands for "Rectal Adenocarcinoma," which refers to cancer that originates in the rectum. COAD and READ are standard abbreviations used in the TCGA (The Cancer Genome Atlas) research project to denote subtypes of colorectal cancer. There is no substantive difference between Figure 1A and 1B for the purpose of the experiment. Both figures aim to demonstrate the expression of DARPP-32 in colorectal cancer tissues. In Figure 1A, the data was obtained from the GEPIA database, which provides a large-scale analysis of gene expression data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) project. This data serves as a reference for the overall expression pattern of DARPP-32 in colorectal cancer. In Figure 1B, the data represents the expression of DARPP-32 in tumor tissues and adjacent normal tissues of 70 colorectal cancer patients from our institution. This data provides a more specific and localized view of DARPP-32 expression in colorectal cancer samples. Together, both figures contribute to the comprehensive understanding of DARPP-32 expression in colorectal cancer, combining both external data from a public database and internal data from our own patient cohort. The redundant description "****P<0.0001" has been removed. Thank you!

17. In Figure 2. Is there any lentivirus or siRNA treatment in the NC group?

In our experiment, the NC group was subjected to empty lentivirus

infection and transfection with negative control siRNA. Thank you!

18. The expression level of DARPP-32 protein was significantly higher in HCT166 cells than that in normal cells (Figure 1H). However, no DARPP-32-positive cells can be apparently observed in the NC group (Figure 3C). Why?

The absence of DARPP-32-positive cells in the NC group (Figure 3C) could be attributed to the fact that the NC group was treated with empty lentivirus and negative control siRNA, which do not induce the expression of DARPP-32. Therefore, the lack of DARPP-32-positive cells in the NC group is consistent with the intended experimental design and confirms the specificity of DARPP-32 expression observed in HCT116 cells (Figure 1H). Thank you!

19. In Figure 2, the cell viability was significantly reduced by about 30% after siRNA intervention. However, there seems to be no significant change in cell density after siRNA intervention for 48 hr (Figure 5C). Why?

The difference in the results observed in Figure 2 (reduced cell viability) and Figure 5C (no significant change in cell density) after siRNA intervention for 48 hours could be due to several factors. Firstly, cell viability and cell density are measurement indicators that reflect different aspects of cell behavior. Cell viability typically assesses overall cell survival and metabolic activity, while cell density measures the

number of cells within a given area. On the other hand, the cell scratch assay involves creating a linear wound (scratch) in a monolayer of cells in a cell culture dish and observing cell migration and wound healing in the scratched area. Since cells have different growth requirements, the cell density needed to achieve confluence in the culture dish also varies. Secondly, the time points chosen for the experiments in Figure 2 and Figure 5C may have different implications for the cellular response. The reduction in cell viability observed in Figure 2 may be an acute effect of the siRNA intervention, indicating immediate cellular response to DARPP-32 knockdown. On the other hand, 48 hours may not be sufficient time for a noticeable change in cell density, especially if the cells have a slow proliferation rate or if compensatory mechanisms are in place to maintain cell density. Thank you!

20. In Figure 6. Please explain why PI3K/AKT pathway rather than MAPK pathway was chosen for further examination in the expression correlation between these proteins and DARPP-32. Moreover, the qualities of some immunoblotting images of PI3K and p-PI3K proteins need to be largely improved.

In our study, we focused on examining the expression correlation between DARPP-32 and specific signaling pathways in colorectal cancer. The choice of investigating the PI3K/AKT pathway instead of the MAPK pathway was based on previous literature and the known involvement of

these pathways in colorectal cancer. The PI3K/AKT pathway and the MAPK pathway are two major signaling pathways involved in cell proliferation, survival, and migration. Both pathways play important roles in various cancers, including colorectal cancer. However, there is evidence suggesting that the PI3K/AKT pathway is frequently dysregulated and associated with tumor progression and resistance to therapy in colorectal cancer. Several studies have reported the involvement of the PI3K/AKT pathway in the regulation of DARPP-32 expression and its downstream effects in different types of cancer (Biochem Pharmacol. 2019 Feb;160:71-79). Additionally, our preliminary data and existing literature indicated a potential correlation between DARPP-32 expression and the PI3K/AKT pathway in colorectal cancer. Considering these factors, we chose to focus on the PI3K/AKT pathway for further examination of the expression correlation between DARPP-32 and specific proteins within this pathway. By investigating the interaction between DARPP-32 and the PI3K/AKT pathway, we aimed to gain a better understanding of the molecular mechanisms underlying DARPP-32-mediated effects in colorectal cancer and potentially identify new therapeutic targets for intervention. It is important to note that our choice of examining the PI3K/AKT pathway does not exclude the significance of the MAPK pathway in colorectal cancer. The MAPK pathway may still play a role in colorectal cancer progression and be

associated with other signaling molecules. Future studies could explore the relationship between DARPP-32 and the MAPK pathway or other signaling pathways to obtain a comprehensive understanding of the molecular mechanisms involved in colorectal cancer development. Thank you!

Reviewer 2# (00607640):

It is my great honor to receive your comments about our paper, and responses are presented below. Thank you very much!

1. Abbreviations used should be with its full name when it firstly appears.

Thank you for your comment. The suggested changes have been made to the revised manuscript.

2. Fig 6E, statistics is recommended.

Thank you for your comment. The statistical analysis for Figure 6E has been performed and the results can be found in Supplementary Figure 1.

3. An editing of English language is recommended.

Thank you for your comment. The suggested changes have been made to the revised manuscript.

Dear editors and reviewers: Thank you very much for your letter and the comments on our paper entitled “DARPP-32 promotes colorectal cancer growth by activating the PI3K/AKT pathway”. We have checked the manuscript and revised it according to the comments and have carefully revised our paper based on the comments of reviewers. The point-to-point responses to the reviewers’ comments are presented below. We also appreciate our dear reviewers for giving us precious advices, which are important for us to improve the quality of our work. Reviewer It is my great honor to receive your comments about our paper, and responses are presented below. Thank you very much! 1. One submitted table contains some non-English characters. Please check it. I apologize for providing a non-English table. I have now made the necessary changes to the non-English parts of the table. Please refer to the attached file. Thank you. 2. The Introduction section should only include the present study's background (relevant research) and experimental purposes. Please omit the last paragraph regarding experimental findings and the conclusion of the present study in the Introduction section. Besides, please also clearly describe the experimental purposes in the Introduction section. Thank you for your comment. We apologize for the description in the introduction and have made relevant changes to the introduction in the revised manuscript (Lines113-114). 3. Please integrate the responses for the significant comments or concerns into the Discussion section and point out the possible limitations regarding the statement "DARPP-32 may be a potential therapeutic target for CRC" in the experimental condition without normal cells included. Thank you for your comment. The suggested changes have been made to the revised manuscript (Lines345-349). 4. According to the description regarding the quantification of qPCR in the Materials and Methods section, DARPP-32 relative expression levels were calculated according to the 2-ΔΔCt method. However, the authors stated that we did not normalize the expression levels of the control group (NCM460 group) to a value of 1. Why? Thank you for your comment. I'm very sorry that my previous answer didn't solve your problem. In this part, when

we use the 2-ΔΔCt method, we calculate the average value of ΔCt of 5 cell lines as a reference value, then calculate the ΔCt value of each cell line and subtract this reference value to obtain ΔΔCt, and finally calculate the ΔΔCt power of 2 to represent the change in relative expression. Therefore, the expression of the NCM460 group is not 1. I apologize for any confusion caused and hope this clarifies the method for you. 5. Please provide the sequence of the negative control siRNA and mention the working concentration of siRNA in the Materials and Methods section. Thank you for your comment. In this experiment, the negative control siRNA used was sourced from a commercial company, RiboBio (Guangzhou, China). Due to commercial confidentiality, the sequence of the siRNA control cannot be provided. The working concentration of siRNA is 20 nM. 6. Why were female mice chosen for the in vivo study? Is it possible that animal menstrual cycles or sex differences interfere with experiments? Thank you for your comment. In this study, our main focus is on the role of DARPP-32 in colorectal cancer tumors. Choosing a single sex was done to avoid the complexities associated with pregnancy in experimental animals. This helps in better control of experimental conditions and reduces the influence of confounding factors. The potential interference of menstrual cycles and sex on the experimental outcomes has been explored in relevant literature (Cancer Cell Int. 2022 May 2;22(1):178; J Biol Chem. 2018 Jul 6;293(27):10606-10619), where researchers have used both female and male mice for nude mouse tumor models. Hence, we speculate that the impact of menstrual cycles and sex on our experimental results is minimal. Based on these considerations, we decided to use female mice for the nude mouse tumor model to investigate the role of DARPP-32 in colorectal cancer tumors. However, we acknowledge that the menstrual cycles and sex-related factors may still have some impact on the study outcomes. Therefore, we encourage future research to validate and support our choice through additional experimental evidence, ensuring the reliability and reproducibility of the study results. 7. Reference #27 still needs the page number. Thank you for your comment. The suggested changes have been

made to the revised manuscript.