Peer Review File

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Reviewer A

My comments are below.

1. Reference should be given for the analyses performed in the method section.
   Reply 1: We have cited references in the analysis section.
2. In the method section, it should be clearly stated which cell lines were used in which analyses. For example, which cells were transfected with siRNA and LV-RhoA lentivirus? What analyses were performed with the transfected cells? Why were only SW480 and SW620 used in the wound healing analysis?
   Reply 2: We have revised your comments in the manuscript
   Changes in the text:
   We performed siRNA transfection using SW480 and SW620 cell lines.
   We performed LV-RNA transfection using SW480 and SW620 cell lines.
   We used SW480 and SW620 cell lines for follow-up experiments.

3. While the information that HIF-1α, RhoA, and ROCK2 expressions were investigated was shared in the "Abstract" and "Quantitative real-time polymerase chain reaction (qRT-PCR)" sections, the results regarding pMYPT1, cyclin D1, and MMP2 expressions were given in the result section. The discussion section explains why the expression levels of these genes were examined and their relationship with the Rho/ROCK2 pathway. The method and rationale for investigating the expression levels of these genes should be included in the abstract, introduction, and method sections. Additionally, what about the p-MYPT1/MYPT-1 ratio?"
   Reply 3: We have revised your comments in the abstract. In this study, we verified that the expression of p-MYPT1/MYPT-1 is determined by the expression of the gray value of Western blot.

Changes in the text: The Western blot results showed that the expression levels of pMYPT1, cyclin D1, and MMP2 in the siRNA + LV-RhoA group were also significantly increased compared with those in the siRNA group.

4. In the results section, it is stated that the experiments were carried out under normoxic conditions and hypoxic conditions, but there is no explanation in the method section about how hypoxic conditions were created. Hypoxia experiment? In addition, which experiments were carried out under hypoxic conditions should be clearly stated in the method section.

Reply 4: We have revised your comments in the manuscript

Changes in the text: Hypoxia culture was performed in a three-chamber air incubator flushed with a gas mixture of 5% CO_2 and 94% N_2 at 37℃. The final O_2 pressure of the medium was measured at a range of 0.5-1%. The above method is widely used to induce hypoxic stress.

5. In the result section, under the title "Suppression of RhoA/ROCK2 signaling pathway significantly inhibited the proliferation and invasion of colorectal cancer cell lines", "normal control group, flank control group, Y-27632 group" are mentioned what this mean should be included in the method section. How was the RhoA/ROCK2 signaling pathway inhibited by Y-27632?

Reply 5: We fully agree with your comment that Y-27632 is commonly used inhibitor of the RhoA/ROCK2 signaling pathway, which we also stated in the abstract.

In cells transfected with LV-RhoA, inactivating the RhoA/ROCK2 pathway with the specific inhibitor Y-27632 decreased tumor growth and metastasis under hypoxic conditions

Reference:

Wang Yuanyuan,Lu Yang,Chai Jixia et al. Y-27632, a Rho-associated protein kinase inhibitor, inhibits systemic lupus erythematosus.[J] .Biomed Pharmacother, 2017, 88: 359-366.

6. The experimental processes by which the results shared in the results and discussion section were obtained should be written clearly and understandably in the method section.

Reply 6: Thank you very much for your comments, we have explained the hypoxia experimental conditions in the methods section

Changes in the text: Hypoxia culture was performed in a three-chamber air incubator flushed with a gas mixture of 5% CO_2 and 94% N_2 at 37℃. The final O_2 pressure of the medium was measured at a range of 0.5-1%. The above method is widely used to induce hypoxic stress.

7. The graphs should be more readable. In Figure 1 C, it is stated that T refers to the tumor, and N refers to normal tissue. What do the repeating Ts and Ns on the horizontal axis (1-6) mean?

Reply 7: Ts and Ns on the horizontal axis (1-6) means tumor and normal tissue.

8. What is the difference between Figure 5 C and D?

Reply 8: Figure 5C and D represent the metastasis and invasion experiments, respectively.

9. "Western blotting indicated a higher protein expression of HIF-1α, RhoA, and ROCK2 in SW480 and SW620 as compared with HCT116 and HT29" Why?

Reply 9: Gray value and bar chart of Western blotting indicated a higher protein expression of HIF-1α, RhoA, and ROCK2 in SW480 and SW620 as compared with HCT116 and HT29.

10. "Moreover, we found that after knockdown of HIF-1α, the RhoA/ROCK2 pathway was inhibited, and the expressions of pMYPT1, cyclin D1, and MMP2 were significantly reduced in the siRNA group compared with the control group."

"Compared with Nomal Control group and Flank Control group, the protein expressions of pMYPT1, cyclin D1 and MMP2 in Y-27632 group were significantly decreased (Figure 5A)".

"The Western blot results showed that the expression levels of pMYPT1, cyclin D1, and MMP2 in the siRNA + LV-RhoA group were also significantly increased compared with those in the siRNA group (Figure 7)."

How were these conclusions reached? Were protein expression levels calculated after western blotting assay? Western blot analysis results should be given as in Figure 1D by calculating protein expression levels.

Reply 10: We very much agree with your comments, we have come to the conclusion that the above is given to the calculation of strip gray values, but the lack of bar chart display, will be adjusted in a future article.

11. In Figure 6, the siRNA + LV-RhoA group and the siRNA group were compared in the SW480 cell line, and the Y-27632 group was compared in the SW620 cell line. Why?

Reply 11: For both sets of experiments, we have validated both sets of cell lines, but only one cell line is shown in the manuscript.

12. "**Lentivirus-mediated overexpression of RhoA** **The lentivirus targeting RhoA** and an empty vector were designed, packed, and purified by GeneChem (Shanghai, China) and used according to the protocol provided by the manufacturer."

"In order to verify whether HIF-1α regulates colorectal progression through the

RhoA/ROCK2 signaling pathway, colorectal cancer cell lines SW480 and SW620 were **transfected with LV-RhoA lentivirus to overexpress RhoA,** while the proliferation, migration, and invasion of the cells were detected with CCK8 and Transwell assays."

"Finally, in 1 order to verify whether HIF-1α promotes the progression of colorectal cancer by regulating the activation of RhoA/ROCK2 pathway, we used siRNA to silence HIF-1α expression while **using LV-RhoA to overexpress the RhoA/ROCK2 pathway inhibitor, Y-27632.**"

If the overexpression of RhoA is targeted by transfection of LV-RhoA with lentivirus, what is meant by the 3rd sentence?

Reply 12: The 3rd sentence is rescue experiment, in which RhoA/ROCK2 pathway inhibitors are added at the same time when RhoA is overexpressed.

13. It is thought that the literature information mentioned in the discussion section does not support the hypothesis of this study. The relevant section can be re-evaluated from this aspect.

Reply 13: Thank you very much for your comments on our manuscript. In the discussion, we mainly discussed the studies of HIF-1α and RhoA/ROCK2 pathways in other directions, while there are few studies in colorectal cancer, which reflects the originality and novelty of our research. The reliability of the results of our study is described, and a new mechanism for the occurrence and development of colorectal cancer is proposed.

Reviewer B

The Authors have carried out a commendable amount of work investigating a potential relationship between HIF-1a and RhoA/ROCK. The work is novel and of interest to the field. The siRNA experiments and the effect on viability, migration and invasion seem robust and very clear with support in Figure 7 that HIF-1a acts upstream of RhoA and ROCK2 (and controls their levels somehow). The ROCK inhibitor experiments are very good as well and Figure 5A shows that the inhibitor indeed hits its targets as judged by reduction of MYPT phosphorylation in both cell lines, even though no IC50 values are mentioned and no concentrations are shown. However, there are a number of experimental issues related to 1) the patient data, 2) the proposed interplay between hypoxia-HIF-1a-RhoA/ROCK (in normoxic and hypoxic conditions), and 3) the RhoA overexpression studies that need to be addressed

before this work is ready for publication.

Major comments

• The patient data is not analysed and presented well enough. The Authors say they have 80 samples, but show results from three samples only – have they performed all analyses on all 80 samples? If yes, please show mean gene and protein expression in samples vs mean expression in normal tissue (with dot plots, paired t-tests, etc). Also, please show the IHC scoring carried out by the two pathologists in a supplementary table for the 80 samples normal vs tumor tissues or make a paired dot plot graph – it is not clear where the percentages of positive samples come from exactly (how are they calculated). It has to be clear if both normal and tumor tissues in the same sample stain positive and in what percentage of samples there is positive staining only in tumor tissues (for example, 27% of normal tissues is positive for ROCK2 and 68% of tumor tissues – does it mean that ROCK2 is upregulated specifically in the tumors of 41% of patients or in some patients normal tissues upregulate ROCK2 compared to tumor tissues?).

Correlation analyses of the expression of HIF-1a, RhoA and ROCK2 and the staging or metastasis or survival data (if available) would strengthen the paper so the Authors can support the argument that these proteins and pathways are important for progression, metastasis and/or survival. Analysis of TCGA datasets can also be helpful to support that point.

Lastly, literature suggests variability in RhoA expression for example so this should be addressed further by the Authors in the discussion – does their patient cohort follow what has been described in other cohorts - doi: 10.1038/bjc.2017.420 .

Reply:Thank you very much for your comments on our manuscript. In order to present clinical information on 80 patients in this study, we have deleted them. In a follow-up study, we will further analyze the data on colorectal cancer in TCGA.

Changes in the text: Specifically, colon cancer and corresponding noncancerous tissues were collected from patients who had undergone radical colectomy but not preoperative chemoradiotherapy.

• There should be Western blots of the LV overexpression of RhoA vs untransfected control cells – there is no evidence by Western Blot or PCR in the current figures that overexpression of the protein (or mRNA) is achieved by the LV vector transfection (the Authors show

siRNA+overexpression). Also, there are no experiments comparing control to LV overexpression in terms of viability, wound healing and transwell migration. This is a major issue related to the proposed mechanisms that activated RhoA and ROCK2 make CRC cells become more aggressive. Also, there is evidence in literature that RhoA/ROCK may regulate HIF-1a levels so a Western blot of HIF-1a in cells that overexpress RhoA would be useful too.

Reply: Thank you very much for your pertinent comments on our manuscript. As for the verification of expression efficiency after overexpression, we have done it in our usual experiments, but we have not put it in the manuscript. In future studies, we will further investigate the in-depth mechanism between HIF-1α and RhoA/ROCK pathways.

• Similarly, there is no comparison between normoxia and hypoxia – do the Authors see more HIF-1a, RhoA, ROCK2 and their targets and more proliferation, migration and invasion upon hypoxia in the two cell lines? This is the main model proposed in the manuscript, but this simple set of experiments (+/- hypoxia) is missing.

Reply: Because HIF-1α is an anoxic factor, we mainly focused on the hypoxic conditions in this study. As you said, the addition of a normal oxygen control will make the results more reliable. We will take your suggestions into account in our future research.

• For all experiments, the Authors should indicate in the methods or figure description if performed in biological or technical duplicates or triplicates.

Reply: In response to your suggestion, we have revised it in the manuscript.

Changes in the text: All experiments in this study were repeated three times.

• Figure 6 is methodologically difficult to explain. The Authors use overexpression of RhoA together with siRNA against HIF-1a as a way of rescue experiments. Why is this combination then not shown for both cell lines?

What is the rationale for comparing “rescued” cells after KD and overexpression to cells treated with the ROCK inhibitor for SW620, and why not do it in SW480 cells too? Also, why not do other combinations as well? Would the ROCK inhibitor still work if RhoA is overexpressed (probably yes as ROCK2 is downstream of RhoA)? Comparing two conditions in one cell line and two other conditions in the other cell lines raises a lot of questions.

If comparing combinations, HIF-1a KD together with ROCK inhibition would make more sense as ROCK inhibition should enhance the effect of the KD.

Reply:For both sets of experiments, we have validated both sets of cell lines, but only one cell line is shown in the manuscript.

• Conclusion – The first sentence about hypoxia and the occurrence and development of CRC

is not supported by any experiments. Even with cell lines, there is no normoxia vs hypoxia comparison of proliferation, migration, invasion.

Reply: We fully agree with your comment that there was a lack of normoxia vs hypoxia comparison in this study. The reason is that this study was mainly focused on hypoxia conditions, so all experiments were done under hypoxia conditions.

• The manuscript may benefit from a summary figure of the model the Authors propose about hypoxia, HIF-1a, RhoA and ROCK2 and the experimental approaches they use (e.g. with the ROCK inhibitor).

Reply: Just as you said that this manuscript lacks summary figure, we will also pay attention to provide a brief and clear summary figure in future manuscript writing.

Minor comments

Figure quality is very poor. Make figures bigger with clear labeling and at least 300dpi resolution. It is almost impossible to evaluate some of the figures (like Figure 5).

Reply: We have uploaded the HD image to the editor.

Please explain in the text why you chose these 2 cell lines and you did not do experiments with the other 2 cell lines.

Reply: We have explained this in the manuscript.

Changes in the text: Western blotting indicated a higher protein expression of HIF-1α, RhoA, and ROCK2 in SW480 and SW620 as compared with HCT116 and HT29 (*Figure 1D*). We used SW480 and SW620 cell lines for follow-up experiments.

Please show IC50 values for the ROCK inhibitor and 2 cell lines and indicate the concentrations used for the experiments. Cell viability can be decreased by anything non-specifically so you should give a lot more details with respect to the inhibitor and cell treatments.

Reply: We have explained this in the manuscript.

Changes in the text: Y-27632(Inhibitors of the RhoA/ROCK2 signaling pathway, 10μmol/ml, 24h)

Please explain how you quantified all migration assay – counting cells manually by eye in several fields? Also was this done in biological duplicates, technical duplicates/triplicates?

Reply: This is a flaw in our manuscript, where we found that the difference between the two sets of experiments could be clearly seen and performed statistical analysis, but the results were not presented in the manuscript. Also, all of our experiments were repeated three times.

Please explain how you quantified the wound healing/scratch assays too – how did you determine the percentage – by eye, with a software?

Reply: This is a flaw in our manuscript, where we found that the difference between the two sets of experiments could be clearly seen and performed statistical analysis, but the results were not presented in the manuscript.

Please explain how RT-qPCR analysis was done (delta CT, delta delta CT or?) – what housekeeping gene was used, how was "relative mRNA expression" determined (relative to what)?

Reply: According to your suggestion, we have added the explanation in the manuscript.

Changes in the text: GAPDH is used as an internal reference gene. Analyze the data according to the $2^{-\Delta\Delta Ct}$ method.

Please explain what "flank control" is exactly. For example in Figure 5 – no "flank control" viability and migration assays are shown, but you have "flank control" in the western blots – please explain why.

Reply: flank control represents the transfection of empty vector virus and is essentially a blank control.

What is the difference between Figure 5 C and D – is it a repeat of the same experiment? If yes, please combine in one figure and indicate that it was performed in biological or technical duplicates.

Reply: Figure 5C and D represent the metastasis and invasion experiments, respectively.

Page 2 Line 13 – "hypoxic stress" induced by siRNA? – clarify/rephrase the sentence

Reply: Thank you for finding my problem. We have revised it in the manuscript.

Changes in the text: With hypoxic stress, small interfering RNA targeting HIF-1α

Line 19 – specify what cells – primary cells or cell lines?

Reply: We're using cell lines for this experiment.

Line 20 – LV together with the inhibitor or both strategies work – clarify the sentence

Specify in the methods section what Y-27632 inhibits (you say specific, but specific for what?)

Reply: According to your suggestion, we have added the explanation in the manuscript.

Changes in the text: we first demonstrated that the activation of RhoA/ROCK2 pathway could be significantly inhibited with Y-27632 (Inhibitors of the RhoA/ROCK2 signaling pathway)

Page 9

Line 23 – please explain what Y-27632 inhibits exactly and give reference to the original paper.

Reply: According to your suggestion, we have added the explanation in the manuscript.

Changes in the text: we first demonstrated that the activation of RhoA/ROCK2 pathway could be significantly inhibited with Y-27632 (Inhibitors of the RhoA/ROCK2 signaling pathway)

line 31-32 – how do you activate RhoA/ROCK2 signaling in your settings (with overexpression of RhoA, right) and how do you show that this promotes tumor progression? Your data does not support this statement also because your IHC scoring system is not correlated to CRC staging or presence of metastasis. Make a clear distinction between in vitro results and clinical data.

Reply: We fully agree with your comment that there is no evidence in our manuscript that LV-A can promote tumor progression, and we will improve this part in future studies.

Page 11

line 3 – "its specific mechanisms" is not clear. Mechanisms of what?

Reply: We fully agree with your comment, and we have added the explanation in the manuscript.

Changes in the text: Although HIF-1α has been reported in colorectal cancer, its specific molecular regulatory mechanisms remain unclear.

Line 7 – "the mechanisms of HIF-1a" – mechanisms by which it does what?

Reply: We fully agree with your comment, and we have added the explanation in the manuscript.

Changes in the text: To investigate the molecular mechanism of HIF-1α in colorectal cancer.

Lines 14-20 – it is not clear what the relevance of P2Y12R,CXCR-4, miR-451b and p300 is to this study? There are other papers on RhoA and ROCK1/2 in CRC or in other cancer types in the context of HIF-1a or migration that can be discussed here.

Reply: We fully agree with your comment, We have consulted a large number of relevant literatures and selected those relevant to this study for discussion.

Line 26 – What is the experimental evidence in the manuscript that RhoA and ROCK2 play a role in the occurrence and development of CRC in patients? If you do the suggested correlation analyses, maybe you can support that statement.

Reply:WB, PCR and immunohistochemistry were performed on clinical tissue samples. The results showed that RhoA and ROCK2 were highly expressed in cancer tissues.

Page 12 – line 4 – please rewrite so it is clear.

Reply: We have rewritten it in the manuscript

Changes in the text: In summary, our study identifies a novel molecular regulatory mechanism in colorectal cancer progression, providing a new target for colorectal cancer patients

• Please explain what “normal control” and “flank control” are (e.g. untreated cells, vehicle, something else) and relabel figures accordingly so it is clear what is meant.

Reply: flank control represents the transfection of empty vector virus and is essentially a blank control.

• The discussion needs to be expanded with comments on the known relationship between HIF-1a and RhoA/ROCK even if in other cancer models. It is possible that HIF-1a regulates RhoA, but it has also been shown that RhoA regulates HIF-1a, which is opposing the proposed model in the manuscript - https://doi.org/10.1073/pnas.1321510111 and https://doi.org/10.1186/s12885-019-6501-8

Reply: We have carefully studied the literature recommended by you, which is of great help to our research. We also have follow-up studies on HIF-1a, in which we will further explore the regulatory relationship between HIF-1a and RhoA/ROCK.

• There are papers on CRC and ROCK1 that should also be addressed in the discussion, especially since the Authors do not give rationale why they tested ROCK2 and did not look at ROCK1 (this should be explained in the introduction – why focus on ROCK2 and not ROCK1).

Reply: We fully understand what you mean, RhoA/ROCK signaling pathway has always been the research direction of our research group, so in this study, we focus on ROCK2.

• Please summarize the experimental evidence that support your proposed model – HIF-1a promotes proliferation, migration and invasion through RhoA and ROCK2 – you have HIF-1a KD that decreases RhoA and ROCK2 protein levels (and respectively some of their downstream targets). But do you have more HIF-1a in hypoxic conditions in the cell lines and do you see more RhoA and ROCK2 (or more pMYPT) then?

Reply: In an experiment to verify that HIF-1a promotes colorectal cancer progression through the RhoA/ROCK signaling pathway, we completed the rescue experiment to validate our conclusions. In addition, the whole experiment was completed under uniform hypoxia conditions, which improved the reliability of our conclusions. For your question, we also agree, we will further design the experiment rigorously in the future research, so as to make our research more perfect.

Reviewer C

The paper titled “Activation of RhoA/ROCK2 signaling by hypoxia-inducible factor 1α in promoting tumor growth and metastasis in human colon cancer” is interesting. this study demonstrated that HIF-1α can promote the growth and metastasis of colon cancer via directly affecting RhoA/ROCK2 signaling and thus represents a novel therapeutic target for colon cancer. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) There is an issue with the order and layout of Figure 1, and Figures 2, 4, and 5 are unclear. Please carefully check the figures and make modifications and replacements.

Reply 1: We uploaded figures to the submission system separately to ensure the consistency and accuracy of the figures.

2) There are many detection methods for cell metastasis and invasion. Why this study only uses crystal violet staining? This result is a bit farfetched. If multiple methods are used, the results may be more reliable. It is suggested to add test results of other methods.

Reply 2:We agree with your comments. In the metastasis and invasion experiment, we adopted the conventional experimental methods of our research group, but the experimental results are reliable. However, in our future experiments, we will adopt various methods to ensure the reliable of our experiments.

3) In the introduction of the manuscript, it is necessary to clearly indicate the knowledge gaps and limitations of prior study and the clinical significance of this study.

Reply 3: We agree with your comments and have made changes in the manuscript.

Changes in the text: However, HIF-1α and RhoA/ROCK2 have not been extensively investigated in colorectal cancer, and their mechanisms of action remain unclear. Further exploration of the in-depth molecular mechanism can provide new insights into the progression mechanism of colorectal cancer and provide new targets and directions for the treatment of clinical colorectal cancer.

4) It is recommended to increase the expression of HIF-1α in patients with colon cancer and compare the clinical pathology.

Reply 4: Immunohistochemical, WB and PCR analyses were performed on tissue samples from clinical patients. We agree with your comments. In subsequent studies, we will add clinical patient pathology and prognostic information, or analyze from TCGA database to enhance the completeness of the story.

5) It is recommended to increase in vivo experiments, and the results may be more convincing.

Reply 5:We agree with you that the weakness of this study is the lack of in vivo experiments. In future studies, we will supplement this experiment.

6) What are the relevant characteristics of the tumor microenvironment of colon cancer? What is the correlation between HIF-1α and the tumor microenvironment? What are the possible goals of future drug development? It is recommended to add relevant content to the discussion.

Reply 6: We agree with your comments and have made changes in the manuscript.

Changes in the text: Hypoxia is an important part of the tumor microenvironment, and HIF-1α, as a hypoxia-inducing factor, plays an important role in tumor progression. Our study also indicates the important role of HIF-1α in the progression of colorectal cancer, providing an important theoretical basis for the treatment of colorectal cancer.

7) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “TRPV3 inhibits colorectal cancer cell proliferation and migration by regulating the MAPK signaling pathway, J Gastrointest Oncol, PMID: 36388700”. It is recommended to quote the articles.

Reply 7: We fully agree with your comments. We have also read a lot of similar literature in manuscript writing and learned their research methods. We will pay attention to these problems in future research and manuscript writing.

8) It is suggested that increasing the functional impact of HIF-1α on apoptosis of colon cancer cells may enrich the study.

Reply 8: Your comments are of great help to our study. In this study, we still lack some experiments to make our study more perfect. In future studies, we will verify the integrity of our study from multiple perspectives.

Reviewer D

The draft of original article titled "ACTIVATION OF RHOA/ROCK2 SIGNALING BY HYPOXIA-INDUCIBLE FACTOR 1α IN PROMOTING TUMOR GROWTH AND METASTASIS IN HUMAN COLON CANCER" by Du et al, is in its very early stage to be considered for publication. I think the author needs to do more, more so that the draft looks like a nice concise written article. I think the draft needs to be re-written.

My specific comments to author are:

1. Please add a proper abstract.

Reply 1: We have included the writing of an abstract in the manuscript.

2. The hypoxic conditions were not explained or referred nowhere in the text (O2 level, incubation time…etc.).

Reply 2: Thank you very much for your comments, we have explained the hypoxia experimental conditions in the methods section

Changes in the text: Hypoxia culture was performed in a three-chamber air incubator flushed with a gas mixture of 5% CO_2 and 94% N_2 at 37℃. The final O_2 pressure of the medium was measured at a range of 0.5-1%. The above method is widely used to induce hypoxic stress.

3. Repetitions must be avoided. There are extremely many repetitions in one sentence, paragraph.

- there are sentences that need to be paraphrased like in:

p5, line 29: ……Total tissues and cell RNA were extracted…

p6, line 9: …… Cells and tissues proteins were …

p6, line 13: …… The protein was separated….

p7, line 33: …… Use GraphPad Prism 5 (…….) to plot…

p8, line 1: …… groups, The t-test …

Reply 3: Based on your comments, we have made changes in the manuscript.

Changes in the text: Total RNA was extracted from tissues and cells using TRIzol reagent.

We used radioimmunoprecipitation with phenylmethylsulfonyl fluoride to extract cell and tissues proteins.

Visualize the data using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

4.Overall figures and graphs are out of focus, as are the graph labels.

Reply 4: We re-uploaded the figures separately into the system.

5. In the IHC, it is not clear where in which tumor section the expression i sassesed: such as parenchyme, stroma…etc.

Reply 5: IHC was approved by a professional pathologist, so it was not marked in the original image.

6. Fig.1: D) doesn't give any information…could go into supplementary as a fig.

Bars represent data from how many experiments?

Reply 6: Fig.1: D Western blot analysis of HIF-1α, RhoA, and ROCK2 in 4 colorectal cancer cell lines, with β- actin used as the internal control.Each experiment was repeated three times separately.

7.Fig.2: is not clear if cells were under normoxic or hypoxic conditions.

Data from how many experiments are shown?
Can't you provide better images with higher magnification?

Reply 7: Fig.2 mainly shows the co-localization of HIF-1α and RhoA in cells, and proves that they may interact, which is carried out under normal oxygen conditions. There are instructions for subsequent experiments under hypoxic conditions. Each experiment was repeated three times, but in the manuscript we show one.

8. Fig.3: are not clear what the hypoxic conditions are, % of O_2, incubation period.
The protein level of HIf1a seems more or less the same for both cell lines under normoxia and hypoxia!!! Differences can be seen only in mRNA levels….
Bars represent data from how many experiments?

Reply 8: Hypoxic conditions are described in the manuscript. There are statistical differences in the data results. Each experiment was repeated three times.

9. Include Fig.7 in the text where the data is discussed (p9).

Reply 9: The results of Fig.7 according to your suggestion are illustrated and discussed in the manuscript.

10. Fig.5: Bars represent data from how many experiments?

To many repetitions in the paragraph p9, line 21-32…analysis/… progression.

Reply 10: Each experiment was repeated three times.

11. Fig.6: Bars represent data from how many experiments?
Exp. conditions: describe?

Reply 11: Each experiment was repeated three times.

12. To many repetitions in paragraph p10, line 4-21…analysis……., migration invasion ability; results;

Reply 12: We agree with your comments that this section is a presentation of the results of the experiment, so there may be repeated words.

13. p.11, line 2-10 rewrite the entire paragraph. Discuss the main finding, do not repeat the results section. Speculate the findings.

Reply 13: We appreciate your comments. In the discussion section, we not only explain the results, but also analyze the shortcomings of previous studies to show the reliability and novelty of our results.

14. p11, line 21-24 rewrite it.

Reply 14: We appreciate your comments, and in the discussion section we restate our

results; Cross-sectional comparison of other studies; The shortcomings and prospects of the research are pointed out. We will design more perfect experiments in the future research.

15. p12, line 1-4 to me is not clear what the meaning of the sentence is?!

Reply 15: In order to verify whether HIF-1α promotes the progression of colorectal cancer by regulating the activation of RhoA/ROCK2 pathway.

Changes in the text: we used siRNA to silence HIF-1α expression while using LV-RhoA to overexpress the RhoA, and RhoA/ROCK2 pathway inhibitor, Y-27632.

16. p12, line 8- specify: providing a new target for…

Reply 16:This study has identified the important role of HIF-1α in the progression of colorectal cancer and identified specific molecular mechanisms. HIF-1α as a target provides a new theoretical basis for the future treatment of colorectal cancer.